ROLE OF CCA1 AND LHY IN THE TEMPERATURE COMPENSATION MECHANISM OF THE CIRCADIAN CLOCK IN *ARABIDOPSIS THALIANA*

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by

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Abstract

Role of CCA1 and LHY in the temperature compensation mechanism of the circadian clock in *Arabidopsis thaliana*

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It has recently been demonstrated that plant performance is enhanced if the internal daily rhythms, generated by the endogenous circadian clock, are in unison with the photoperiodic cycle of the environment. The clock is known to be well buffered against daily and seasonal temperature changes, i.e. the clock is temperature compensated. Keeping in mind the adaptive significance of a tuned and well buffered clock, it is important to understand the mechanism underlying the temperature compensation process, as well as to explore the link between the circadian clock, plant performance and its environment.

Transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LONG ELONGATED HYPOCOTYL (LHY), are key components of the *Arabidopsis* circadian clock which have been shown to play different roles in temperature compensation despite their high molecular similarities and widely accepted functional redundancy. Mechanisms of regulation for CCA1 and LHY are largely unknown, hence this study aims to gain an insight into whether the differences between CCA1 and LHY involve differences in promoter-mediated regulation. The two promoters were swapped between the two genes to produce *CCA1::LHY* and *LHY::CCA1* constructs, which were introduced into *lhy* and *cca1* nulls, respectively. The new plants were monitored at 17°C and 27°C to examine the temperature effect on the promoters. Indeed, *CCA1* and *LHY* are regulated differently and this regulation is temperature dependant. While the circadian phenotype of the *LHY::CCA1* transgenic plants mimicked the wild type at both temperature treatments, it was highly impaired in *CCA1::LHY* mutants at 17°C and completely abolished at 27°C. Therefore, differences between *CCA1* and *LHY* are likely to occur due to their divergence in cis-regulation.

To examine if differences in circadian responses were due to altered levels of *CCA1* and *LHY*, plants with extra copies of each gene were created. This showed that the circadian system is highly buffered against transcriptional changes, as up-regulation of either gene did not disturb the circadian clock. However, comparison of plants under 27°C conditions revealed that plants with extra *LHY* displayed longer period, suggesting that LHY is likely to be involved in a mechanism prolonging circadian period at high temperatures and thus contributing to the temperature compensation of *Arabidopsis*.

Natural variation in temperature compensation of the circadian clock was examined amongst accessions from different geographical locations using leaf movement and expression of *CCA1* and *LHY* genes. This revealed that despite a variable leaf movement response to elevated temperature, all accessions experience period shortening in gene expression at 27°C. Accessions with the most temperature compensated gene expression are more temperature tolerant, as identified by a growth performance test. An accession-specific temperature limit exists above which uncoupling of circadian loops occurs, as shown by temperature induced variation in circadian period between the outputs. It is concluded that CCA1 and LHY contribute differently to temperature compensation, and both are important components of this mechanism. Their proper functioning is likely to be of adaptive significance to ensure optimal plant performance in a variable environment. The practical application of these findings so that crops are adapted optimally will be of increasing importance in the context of continuing increases in the human population and possible global warming.

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Abbreviations

Asp – Asparatic acid

ATP – Adenosine 5'-triphosphate

Ca - Calcium

CaMV – Califlower mosaic virus

CAB – CHLOROPHYLL A/B BINDING PROTEIN

CAT – CATALASE 3

CCA1 – CIRCADIAN CLOCK ASSOCIATED 1

CCD – Charge-coupled device

CCR2 – COLD CIRCADIAN RHYTHM RNA BINDING

CHE – CCA1 HIKING EXPEDITION

CK2 – Casein kinase 2

CO – CONSTANTS

DD – Constant Dark

DF – Delayed fluorescence

DNA – Deoxyribonucleic acid

EE – Evening element

FFT – NLLS – Fast fourier transform nonlinear least squares

FLC – FLOWERING LOCUS C

FRI – FRIGIDA

FRQ – FREQUENCY

GI - GIGANTEA

GUS – Beta-glucuronidase

Kan – Kanamycin

LD – Light-Dark cycle

Lhcb – LIGHT HARVESTING CHLOROPHYLL A/B

LHY – LATE ELONGATED HYPOCOTYL

LL – Constant Light

LOV – Light, oxygen or voltage

LUC – firefly *LUCIFERASE*

mRNA – messenger ribonucleic acid

MS – Murashige and Skoog

ORF – Open reading frame

OX – Overexpression

PCR – Polymerase chain reaction

PIF3 – PHYTOCHROME INTERACTING FACTOR 3

PSII – Photosystem II

PRR – Pseudo response regulator

QTL – Quantitative Trait Loci

RAE or Rel. Amp. Error – Relative amplitude error

RNA – Ribonucleic acid

SE – Standard error

T – transformant

UTR – Untranslated region

TOC – TIMING OF CAB

WC – WHITE COLLAR

WCC – WHITE COLLAR complex

WT – Wild type

ZTL – ZEITLUPE

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Chapter 1 - Introduction

1.1. Circadian rhythms

Many biological processes within an organism follow a rhythmical pattern. The period length of a biological rhythm can range from less than a minute (e.g. defecation of *C. elegans*) to years (e.g. animals migration) (Liu and Thomas, 1994; Dunlap, 1999). Rhythms that are synchronized with the day:night cycle and have a period of approximately 24-h are called diurnal (Aschoff, 1963). However, the environment is not constant and a lot of variation in environmental parameters such as light, temperature, humidity, etc. occurs on a day to day basis as well as seasonally. Despite the variation in the environmental time cues and when these time cues are completely absent, some biological processes continue to oscillate rhythmically. For example, the humans sleep/awake cycle persists even in the absence of the day:night alternations (Mills et al., 1974), and the circadian leaf movement in plants also continues when grown under continuous dark conditions (deMairan (1729) in McClung, 2006). To ensure that biological and physiological rhythms in living organisms do not stop in the temporal absence or variation in the environmental variables, many processes are under the control of an endogenous oscillator or "the clock", and the biological rhythms it controls are called circadian (Latin "about a day") (Aschoff, 1963; Bünning (1960) in McClung, 2006). Circadian rhythms are driven by the Earth's rotation on its axis and hence have a period of approximately 24 h , which persist even in the absence of the environmental stimulus (Figure 1.1) (Dunlap, 1999; Harmon et al., 2005).

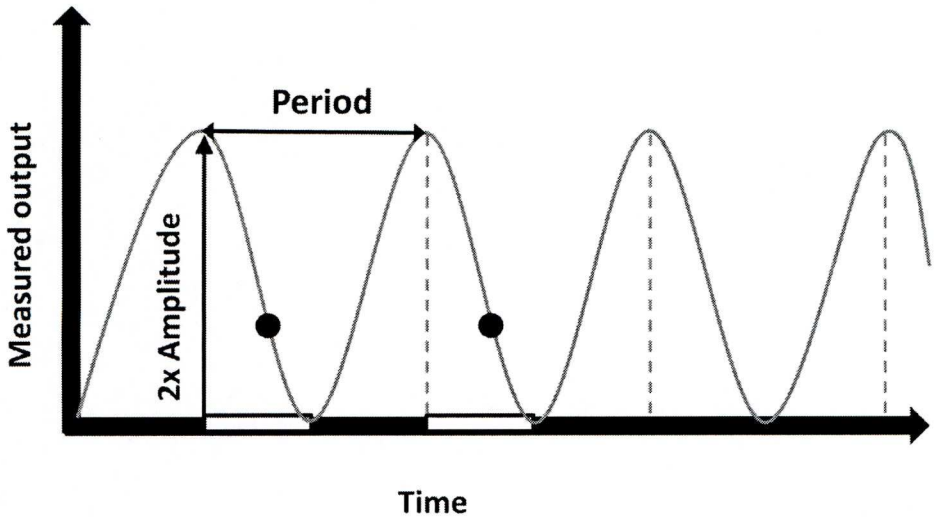


Figure 1.1. Plot and terms describing circadian rhythms.

X-axis is time in hours where white sections of the axis correspond to day time, black sections to night, and continuous black section is continuous dark. **Period** is usually described as time from peak to peak, while **amplitude** is a half of the distance between peak and trough. **Phase** is defined as time within a cycle for a particular event and is depicted by black circles.

Circadian rhythms are found in eukaryotes and some prokaryotes (e.g. cyanobacteria) and include biological processes such as leaf and petal movement in plants, conidiation in fungi, sleep/wake cycle in humans and nitrogen fixation in bacteria (Pittendrigh et al., 1959; Kondo et al., 1993; McClung, 2001; Albrecht, 2002). In mice, a mammalian model system, up to 10% of genes, sampled from any tissue type, have been shown to be circadian regulated (reviewed in Lowrey and Takahashi, 2004). In *Arabidopsis thaliana* between 6 to 16% of the genome is under direct circadian control, as identified by several microarray analyses (Harmer et al., 2000; Edwards et al., 2006). Moreover, a luciferase enhancer trapping technique revealed that up to 36% of the *Arabidopsis* genes are circadian mediated, further supporting the importance of circadian rhythms (Michael and McClung, 2003).

Circadian regulation of endogenous physiological processes is especially important to sessile organisms, including plants. These organisms are highly dependent on their environment as physical escape from perturbations is rarely possible. It is an adaptive advantage to have an endogenous rhythm that matches periodic environment (Ouyang et al., 1998; Dodd et al., 2005). For example, deviation of the photoperiod from the 24 h cycle negatively affects growth of tomato plants, and prolonged exposure of these plants to continuous light significantly damages them (Highkin and Hanson, 1954). Another example is cyanobacteria, *Synechococcus* spp., where mutants with free-running periods of 22 h and 30 h out-competed each other and the wild type when grown under cycles of 11:11 L:D (light:dark) and 15:15 L:D, respectively (Ouyang et al., 1998). Furthermore, *Arabidopsis thaliana* short and long period mutants performed better when their circadian clocks matched the external L:D environment (Dodd et al., 2005). For instance, *toc-1*, a short period mutant, fixed more carbon, accumulated more

vegetative biomass and showed increased survival rates in comparison to the wild type control, when grown under 10:10 L:D conditions. In contrast, *ztl-1*, a long period mutant, performed better and outcompeted *toc-1* and the wild type, when it was grown under long days (Dodd et al., 2005).

It is important to notice that circadian rhythms are well compensated against temperature perturbations in the environment (Pittendrigh, 1954). Even though the rate of biochemical processes is known to at least double over a 10°C temperature change, the period of a temperature compensated rhythm stays approximately the same (Zimmerman et al., 1968; Somers, 1999; Ruoff and Rensing, 2004). The first proof of temperature compensated circadian rhythms was obtained by Pittendrigh (1954), who tested circadian *Drosophila* (fruit fly) pupal emergence at different temperature conditions. The fly eclosion took place early in the morning at 24 h periodicity regardless whether it was grown at 16, 21 or 26°C. Confinement of the event to dawn, when it is cold and wet, is accepted to be of high adaptive value, as the new flies can expand their wings only at high humidity.

A well temperature compensated clock allows organisms to keep track of time regardless of temperature changes in the environment, as long as it stays within their physiological range (McClung, 2006). On the other hand, pulses of temperature as well as other stimuli (e.g. light or dark pulses) can affect a rhythm by changing its phase and consequently reset the clock (Aschoff, 1963; Somers et al., 1998; Devlin, 2002; Rensing and Ruoff, 2002). These environmental stimuli are called Zeitgebers (Halberg et al., 1959 in Aschoff, 1963). Zeitgebers are essential in setting a rhythm to the correct time of the day as well as to coordinate the clock according to the seasonal changes throughout the year (Figure 1.2 A).

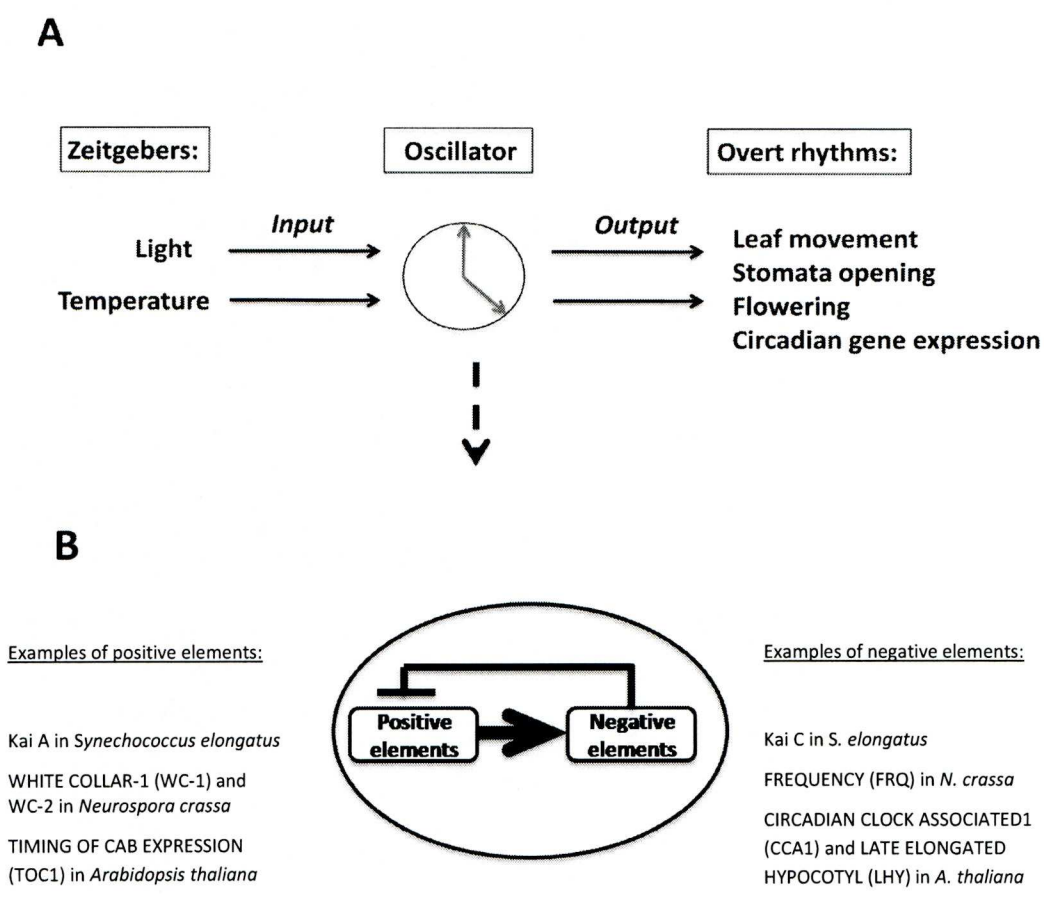


Figure 1.2. Generalized representation of a circadian system.

A – Circadian clock divided into 3 parts: 1) Zeitgebers, any stimulus involved in entraining the clock; 2) central oscillator, and 3) circadian outputs (overt rhythms)

B – A schematic depiction of interlocking positive and negative feedback elements composing the central oscillator.

Therefore, the main characteristics describing circadian rhythms are:

- Circadian rhythms are self-sustained and oscillate with approximately 24 h period even in the absence of a stimulus
- Circadian rhythms are temperature compensated
- The phase of circadian rhythms can be altered with an appropriate stimulus, for example, a temperature or light/dark pulse

1.2. The circadian clock

The general circadian clock model consists of 3 main parts: input pathways, core oscillator and output pathways mediating overt rhythms (Figure 1.2 A) (Somers, 1999; Harmer, 2009). The input pathway is associated with entrainment signalling, and is linked to output pathways via the central oscillator (Somers, 1999). The central oscillator is a combination of positive (which promote activity) and negative (inhibit activity) elements composing interlocking negative/positive feedback loops (Figure 1.2 B) (Dunlap, 1999). In eukaryotes these loops commonly consist of interacting genes and proteins. Such transcription/translation feedback loops make up the basis of eukaryotic circadian clocks (Dunlap, 1999). However, the identified clock genes of transcriptional feedback loops are not conserved across taxa and, therefore, it is most likely that they have evolved independently (Johnson and Kyriacou, 2005). The circadian clock has been most extensively studied in several model systems i.e. *Synochoccoccus elongatus* (cyanobacteria), *Neurospora crassa* (fungus), *Drosophilla melanogaster* (fly) and mice (Golden et al., 1997; Young and Kay, 2001; Panda et al., 2002; Lowrey and Takahashi, 2004). Circadian systems of *Synochoccoccus* and *Neurospora* will be described further on. The current work has

been carried out in the plant model organism, *Arabidopsis thaliana*, whose circadian clock will be presented in more detail.

1.3. Circadian clock in cyanobacteria

The general features of circadian rhythms are the same across various taxa, however, the underlying molecular mechanism controlling the rhythms is different. The simplest circadian oscillator that has been described is of the cyanobacteria *S. elongatus*, where transcription of essentially all genes is under circadian control (reviewed in Johnson et al., 2008a). The main mechanism of the *S. elongatus* clock is comprised of three proteins – KaiA, KaiB and KaiC (Ishiura et al., 1998). Even though interaction between all 3 proteins drives the global rhythms of *S. elongatus*, phosphorylation of KaiC, required for such interaction, oscillates with a 24 h rhythm independently of KaiABC transcription. Interestingly, after transfer to continuous dark (DD), where the rhythmic transcription/translation of Kai proteins was abolished, the phosphorylatated state of KaiC continued to oscillate, and was temperature compensated (Tomita et al., 2005). Furthermore, incubation of isolated KaiA, KaiB and KaiC proteins supplemented with ATP (adenosine 5'-triphosphate) *in vitro*, resulted in a 24 h circadian rhythmicity in KaiC phosphorylation (Nakajima et al., 2005). The period rhythmicity of KaiC phosphorylation stayed relatively unchanged at different temperatures confirming the temperature independent nature of generated rhythms (Nakajima et al., 2005). This discovery suggests that circadian rhythms do not always require the presence of transcriptional/translational negative feedback loops, but could be generated via the post-translational oscillator (Tomita et al., 2005; Johnson et al., 2008b). Overall, analysis and understanding of primitive clocks from prokaryotic organisms provides important information on the fundamentals of circadian oscillators as well as their further evolution.

1.4. Circadian clock in *Neurospora crassa.*

1.4.1. Circadian clock

The early molecular basis of eukaryotic circadian clocks originated from the model system of *Neurospora crassa*, a filamentous fungus. This organism has also been a good model in understanding the mechanism of temperature compensation in circadian rhythms. In the late 50's it was noticed that the formation of asexual spores (conidiospores) in *N. crassa* was under circadian control (Pittendrigh et al., 1959) and in the 1970s, the first clock gene, *FREQUENCY* (*FRQ*), was identified (Feldman and Hoyle, 1973). *FRQ* mRNA, FRQ protein together with 2 transcription factors, WC-1 (WHITE COLLAR-1) and WC-2 (WHITE COLLAR-2) are the core components of the circadian clock of *Neurospora* (Crosthwaite et al., 1997). In this system, FRQ is a negative element that inhibits the activity of WCC (White Collar complex) positive elements, consisting of WC-1 and WC-2 (Figure 1.3). Levels of *FRQ* mRNA and FRQ protein oscillate throughout the day, while amounts of *WC-1* and *WC-2* RNA stay relatively unchanged (Dunlap, 1999; Lee et al., 2000). Late in subjective night WCC binds to the *FRQ* promoter and activates its transcription. On the other hand, FRQ protein interacts with the WCC later in the day and, by blocking WCC activity, inhibits its own transcription. Low levels of *FRQ* mRNA lead to the decrease in FRQ and consequently to the release of WCC blockage, which reactivates *FRQ* transcription to start a new cycle (Dunlap and Loros, 2004). Loss of the proper functioning of the FRQ, WC-1 or WC-2 leads to a change in the period phenotype and inability to temperature compensate the rhythm. In addition, disruption of *FRQ* mRNA oscillation by its overexpression causes arrhythmia (Aronson et al., 1994; Liu and Bell-Pedersen, 2006).

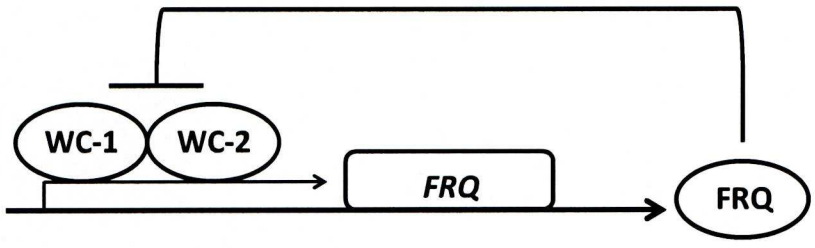


Figure 1.3. A simplified model of *N. crassa* circadian positive/negative feedback loop.

WC-1 and WC-2 form a complex (WCC) which binds to the promoter of the *FRQ* gene activating its expression. FRQ protein in its turn represses activity of WC-1 and WC-2. Genes are boxed, while proteins are encircled.

1.4.2. Temperature compensation

The mechanism of temperature compensation has been well studied in *N. crassa* (reviewed in Brunner and Diernfellner, 2006). The temperature range permissive for circadian rhythmicity in *Neurospora* ranges from approximately 15°C to 34°C (Gardner and Feldman, 1981). The main role in temperature compensation is played by FRQ (Liu et al., 1997; Diernfellner et al., 2005). There are 2 forms of FRQ present in *Neurospora*; long form – $FRQ^{1\text{-}989}$ and short form – $FRQ^{100\text{-}989}$, which differ by 100 amino acids (Garceau et al., 1997). Even though either form is sufficient to maintain a rhythm at 25°C (with a reduced quality) in the wild type background, the ratio of the two forms, as well as the total amount of FRQ, is changeable and is temperature dependant. Liu et al. (1997) showed that mutants expressing only the long form of FRQ are rhythmic at 30°C i.e. the upper limit of temperature permissive for *Neurospora* circadian rhythmicity, however they become arrhythmic at 20°C. On the opposite side, mutants with only the short FRQ are rhythmic at low temperatures, but lose their rhythm past 27°C. Taken together, depending on the form of the FRQ, each mutant performs better at the certain temperature extreme.

The ratio of L (long) versus S (short) forms of FRQ is controlled by the splicing of intron 6, where the translation initiation site of L-FRQ is (Colot et al., 2005; Diernfellner et al., 2005). This splicing event is temperature regulated, with more intron 6 being spliced out at low temperature leading to less L-FRQ and consequently lower L-FRQ/S-FRQ ratio (Colot et al., 2005). Interconnected with temperature controlled regulation of FRQ forms, another temperature dependent mechanism is present in *Neurospora*, which drives the total amount of FRQ. This mechanism involves 6 open reading frames (uORFs) in the 5'-UTR upstream the

main ORF encoding FRQ, where scanning ribosomes can be trapped/stalled, most likely leading to dissociation, which causes an overall decrease in the FRQ translation (Diernfellner et al., 2005). The higher the temperature, the less efficiently uORFs are translated, thus increasing the efficiency of the FRQ translation, which results in higher levels of total FRQ (Diernfellner et al., 2005).

Overall, the remarkable mechanism of controlling the total amount of FRQ and changing the ratio of FRQ forms makes up the basis of the temperature regulation mechanism in *Neurospora*, and results in an optimal clock with buffered period and amplitude rhythms over a wide range of temperatures (Liu et al., 1997; Diernfellner et al., 2005; Brunner and Diernfellner, 2006).

1.5. Monitoring circadian rhythms in plants

1.5.1. Leaf movement assay

First circadian rhythms were recorded in plants in the beginning of the 18th century, when Jean Jacques Ortous de Mairan noticed that leaves of the sensitive plant, changed their positions according to the time of the day and the movement persisted even in continuous darkness (McClung, 2006 and references therein). Since then, monitoring leaf movement has been widely used to assess circadian rhythmicity in many plants (Bunning, 1964), including the model plant, *Arabidopsis thaliana* (Edwards and Millar, 2007). Leaf movement assay involves a collection of a series of plant images taken over a period of time at regular intervals (Engelmann et al., 1992). The current, commonly used leaf movement system was based on the Kujata digital imaging system (Dowson-Day and Millar, 1999), and was described in detail in Edwards et al. (2005). The system consists of CCD (charge coupled device) video cameras, which are placed inside a growth chamber to allow careful selection and control of temperature and light. *Arabidopsis* (or other plants) seedlings,

previously entrained to an appropriate photoperiod, are positioned in front of video cameras and are left for a period of several days to grow under continuous light, where plant images are taken every 20 minutes. The position of leaves or cotyledons in these images is later analyzed to determine a free-running period of circadian leaf movement. For that, plant generated oscillations are plotted against time, and their fit to the cossin wave is analysed using the Fast Fourier Transform Nonlinear Least Squares program (FFT – NLLS) which, at the end of the analysis produces calculated period values (Edwards and Millar, 2007).

1.5.2. Luciferase assay

In addition to leaf movement, other physiological processes such as hypocotyl growth, flowering, photosynthesis, delayed fluorescence and cytosolic free Ca^{2+} concentrations have also been shown to be under circadian control (Johnson et al., 1995; Dowson-Day and Millar, 1999; Suarez-Lopez et al., 2001; McClung, 2006; Gould et al., 2009). Moreover, up to 36% of the whole *Arabidopsis* transcriptome is thought to be circadian regulated (Harmer et al., 2000; Michael and McClung, 2003). Monitoring gene expression profiles in *Arabidopsis* has become easier with the introduction of the luciferase bioluminescence assay (Millar et al., 1992). Luciferase (LUC) is an enzyme found in several organisms, including the firefly, *Photinus pyralis*, which is the most commonly used in laboratories (Alam and Cook, 1990). Upon oxidation of luciferin, catalyzed by luciferase, a photon of light is emitted and can be detected using sensitive video cameras (Alam and Cook, 1990). Fusion of the luciferase coding region to the promoter of a gene of interest allows non-destructive assay of gene expression in real time. The luciferase bioluminescence assay involves transforming an organism, for example *Arabidopsis* plants, with a reporter construct consisting of the *LUC* gene fused with a selected gene promoter (Millar et al., 1992).

Before the experiment, plant transformants are sprayed with luciferin and the next day are transferred into the imaging chamber. The imaging chamber holds a CCD (charge coupled device) camera which is mounted to the top of a growth incubator. The camera is controlled by a special software which is programmed to take images at certain time intervals. Analysis of these images is later performed where the amount of light emitted from a plant or a group of plants is quantified (Hall and Brown, 2007). The obtained values can then be plotted against time and, in the case of circadian experiments, the period of oscillations in light emission can be calculated. The luciferase biolumenescence method has been an exceptional tool when characterizing circadian clock mutants, including the first *Arabidopsis* clock mutant, called "*timing of CAB expression 1*" (*TOC1*) (Millar et al., 1995).

1.5.3. Delayed fluorescence assay

Recently, the light sensitive camera, used for the bioluminescence assay, has been adapted for monitoring delayed fluorescence in plants, including *Arabidopsis* (Gould et al., 2009). Delayed fluorescence (DF) is a discharge of excess photons left over from the excitation of the photosystem II (PSII) (Rutherford et al., 1984), and subsequently that light discharge can be quantified using exactly the same setup as in the bioluminescence assay. The only major difference between the assays is that, due to the rapid decay of DF, the camera has to be programmed to acquire images immediately after plant exposure to light (Gould et al., 2009). Conveniently, the wavelength of light emitted by delayed fluorescence and luciferase is different, allowing simultaneous measurement of 2 circadian outputs in the same plant. Another important advantage of DF application is that this system can be readily used with plants, whose circadian assessment is limited due to low transformation success (Gould et al., 2009). Overall, DF assay provides a non-distructive high-

throughput technique, potentially applicable to all photosynthetic organisms.

1.5.4. Measuring multiple outputs

Measuring different outputs of the circadian clock resulted in an interesting observation that circadian periods of different outputs, generated by the same plant, do not always match (Hall et al., 2002; Thain et al., 2004; Gould et al., 2009). This data indicates that multiple circadian oscillators are present in a plant, generating rhythms of different periods. While some researchers propose that such multiple clocks can function autonomously holding tissue-specific properties, others suggest coupling of multiple circadian outputs to the same molecular oscillator (Thain et al., 2000; James et al., 2008). A high correlation between periods generated from different rhythmic outputs of a circadian mutant supports the latter idea.

1.6. *Arabidopsis* circadian clock

For a long period of time, leaf movement and plant growth rhythms were the ultimate source of information on the circadian clock (Bunning, 1964; McClung, 2006). However, at the end of the 20th century, with the help of a forward genetic approach, the dissection of the central oscillator and the identification of its components became possible. The first plant clock mutant was identified from a screen of mutagenized *Arabidopsis* lines transformed with a *LUC* reporter gene fused to the *Lhcb* (*LIGHT HARVESTING CHLOROPHYLL A/B PROTEIN)* promoter (Millar et al., 1995). This mutant was called "*timing of CAB expression*" or *toc1*, and had a shortened period of 21 h when under continuous light (LL). Assuming that endogenous oscillations were generated by interlocked feedback loops, the search continued for negative elements of the *Arabidopsis* circadian clock. CCA1 (*CIRCADIAN CLOCK ASSOCIATED1*) was first isolated as a protein binding to the *Lhcb* gene and activating its expression via the phytochrome transduction pathway

(Wang et al., 1997). At the same time, LHY (*LATE ELONGATED HYPOCOTYL*) was identified in a flowering screen of mutants containing a Ds transposon element overexpressed from the *CaMV* 35S promoter (Schaffer et al., 1998). *LHY*-overexpressor flowered much later than its wild type background when grown under long day conditions (16:8 L:D). Like CCA1, LHY has also been shown to bind to the *Lhcb* promoter (Schaffer et al., 1998; Kim et al., 2003).

According to the current model of the *Arabidopsis* circadian clock, three transcriptional feedback loops (main, evening and morning) make up the central plant oscillator (Figure 1.4) (Locke et al., 2005a; Locke et al., 2006). These loops and their members will be discussed below in more detail.

1.6.1. The main clock loop

The main loop in *Arabidopsis* was first described in 2001 and included 3 members: CCA1, LHY and TOC1 (Alabadi et al., 2001; Gardner et al., 2006). TOC1 corresponds to WC1 and WC2 in *Neurospora*, while CCA1 and LHY function could be compared to FRQ (Figure 1.2 B). CCA1 and LHY are morning expressed MYB-like transcription factors, which directly bind to the *TOC1* promoter inhibiting its expression (Alabadi et al., 2001). TOC1, on the other hand, is a positive regulator of *CCA1* and *LHY* expression. Therefore, a decline in TOC1 levels leads to a decline in *CCA1* and *LHY* expression and a subsequent release of the inhibition of *TOC1* expression peaking in the early evening (Alabadi et al., 2001; Carre and Kim, 2002; Gardner et al., 2006). TOC1 does not directly bind to the promoters of *CCA1* and *LHY*, and its action is somehow mediated via an unknown component X (Figure 1.4, central loop) (Locke et al., 2005b). Recently it has been suggested that a CHE (CCA1 HIKING EXPEDITION) protein could play a partial role of the X function (Pruneda-Paz et al., 2009).

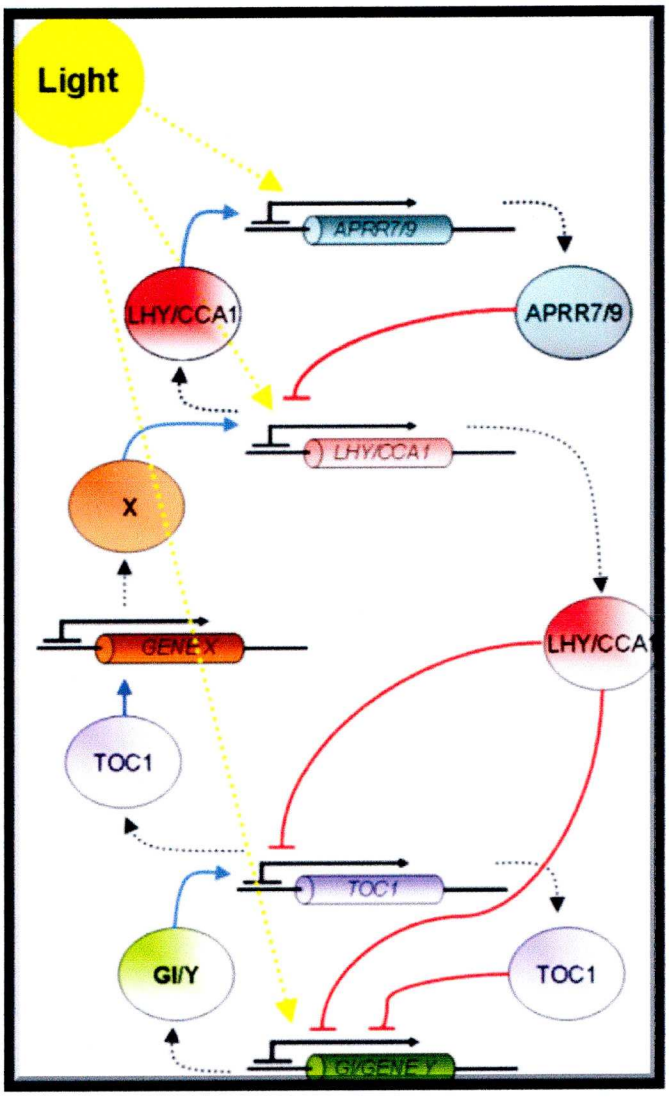


Figure 1.4. A simplified three loop model of the *Arabidopsis* circadian clock (adapted from Locke et al., 2006).

In the main loop, CCA1 and LHY inhibit expression of *TOC1*, while TOC1 promotes expression of *CCA1* and *LHY*. The morning loop consists of APRR7/PRR7 and APRR9/PRR9 repressing expression of *CCA1* and *LHY*, and CCA1 and LHY activating expression of *APRR7* and *APRR9*. The evening loop is made up by GI, a positive regulator of *TOC1* expression, and TOC1, a negative regulator of *GI*. Arrowed lines indicate positive function, whereas lines with a perpendicular ending represent inhibitive function. Yellow arrows indicate light induction. Proteins and genes are depicted as circles and cylinders, respectively.

1.6.2. Clock related gene – *TOC1*

TOC1 is a member of a pseudo response regulators (PRR) family (Makino et al., 2002). A receiver domain is present on the N-terminus of the TOC1 protein, which is known to sense and transduce various environmental signals. However, several substitutions are present in the sequence of the TOC1 receiver domain, including a conserved asparatic acid (Asp) essential for its functioning as a response regulator (Strayer et al., 2000). Despite that, TOC1 is a functioning protein and according to new studies regulation by TOC1 is most likely mediated through its interaction with other proteins (Pruneda-Paz et al., 2009). *TOC1* mRNA levels oscillate rhythmically and mutations in *TOC1* disrupt the circadian phenotype of *Arabidopsis* (Strayer et al., 2000; Mas et al., 2003a). Over-expression of *TOC1* results in arrhythmicity of circadian outputs, and loss-of-function *toc1* mutants have a short period under continuous light (LL) and disrupted rhythms in continuous darkness (DD) and red light (Mas et al., 2003a). In addition, high levels of *TOC1* correlate with an increased sensitivity to light, resulting in a very short hypocotyl length of *Arabidopsis* plants, whilst *toc1*-null's hypocotyl is longer. Overall, this suggests that *TOC1* not only plays a part of the circadian transcriptional feedback loop, but is also involved in the light signal transduction to the circadian clock itself.

1.6.3. Clock related gene – *ZTL*

Levels of TOC1 protein also oscillate in a circadian manner and are mediated through interaction with ZTL (ZEITLUPE) (Mas et al., 2003b). Like *toc1*, the *ztl* mutant was identified from a mutagenized *Arabidopsis* carrying *Lhcb::LUC* gene reporter (Somers et al., 2000). ZTL is an F-box protein carrying 6 kelch repeats and a LOV (Light, Oxygen or Voltage) domain (Mas et al., 2003b; Somers et al., 2004). LOV domains can bind the flavin chromophore and therefore is suggested to be

linked with the light-dependent function of ZTL. Acting as a blue light photoreceptor, ZTL mediates the light input to the circadian clock. Blue light enhances the interaction between ZTL and GIGANTEA (GI) consequently stabilizing its own degradation. Due to the rhythmic expression of GI, the ZTL-GI interaction oscillates, conferring post-translational regulation of ZTL in a circadian manner (Kim et al., 2007). Consequently, rhythmic regulation of ZTL results in rhythmicity in ZTL interaction with other proteins, including targeting TOC1 for proteosomal degradation. As a result, plants lacking functional ZTL have a long-period, a phenotype achieved by the disruption of ZTL and TOC1 interaction, where consequent accumulation of TOC1 protein prolongs circadian period (reviewed in Hanano and Davis, 2005).

1.6.4. Clock related genes – *PRR*s and the 2nd clock loop

There are 4 other genes in *Arabidopsis* which result in proteins with a function similar to TOC1 (Makino et al., 2002; Nakamichi et al., 2005), these are PRR3, 5, 7 and 9. TOC1 was originally named PRR1. Expression of these genes is synchronized and starts with *PRR9* peaking at dawn, followed by *PRR7*, *PRR5*, *PRR3* and *TOC1* with 2 h intervals between each other (Matsushika et al., 2000). Except for *toc1*, none of the *prr* single mutants have a strong circadian phenotype, suggesting that they function outside the main loop of the *Arabidopsis* circadian clock. Consequently, 2 more interconnected feedback loops have been proposed apart from the central TOC1-CCA1/LHY loop: morning-phased and evening-phased (Figure 1.4) (Locke et al., 2006). Data suggests that PRR7 and PRR9 together with CCA1 and LHY make up a morning circadian loop, where PRR7 and 9 negatively regulate *CCA1* and *LHY* and, in return, CCA1 and LHY promote expression of *PRR7* and *PRR9* (Figure 1.4) (Farre et al., 2005; Zeilinger et al., 2006; Farre and Kay,

2007). Both *prr7* and *prr9* nulls have a longer circadian period than the wild type and this effect is light dependant. *prr7* has a long period under continuous red or blue light, with red light having a stronger effect on the period (Farre et al., 2005). On the other hand, circadian period of the *prr9* is similar to the wild type when assayed under continuous red light or DD, but the period is prolonged under continuous blue light (Eriksson et al., 2003). In addition, *prr7:prr9* double mutant could not be entrained to thermocycles, suggesting that the two genes are involved not only in light but also in temperature perception (Salome and McClung, 2005).

1.6.5. Clock related gene – *GI* and the 3rd clock loop

The third, evening loop of the main *Arabidopsis* circadian oscillator, involves positive/negative interaction between GI and TOC1 (Locke et al., 2006). *gi* was first identified as a late flowering mutant from an *Arabidopsis* screen mutagenized with X-rays (Rédei, 1962). It was later confirmed, that GI is involved in the flowering mechanism, functioning upstream from *CONSTANTS* (*CO*), a flowering time gene (Fowler et al., 1999). Expression of *GI* is under circadian control and GI protein has a general effect on circadian rhythms, by regulating the accuracy of the circadian clock (Mizoguchi et al., 2005). GI has also been shown to play an important role in the temperature compensation mechanism of the *Arabidopsis* circadian clock (Gould et al., 2006). The g*i* loss-of-function mutant exhibits a temperature dependant phenotype, with plants losing circadian period robustness when temperature fluctuates away from the norm. In addition, the free-running period of *gi* mutants, measured by leaf movement and *CAB::LUC* reporter gene assays, was substantially shorter when assayed at high or low temperatures in comparison to the wild type, but did not differ from the control when measured at normal temperature (17°C). Further research proposed that the presence of functional GI and LHY ensured the

period change with increasing temperature is minimal. While the balance between GI and CCA1 is important in cool temperatures (Gould et al., 2006).

1.6.6. Clock related genes – *CCA1* and *LHY*

CCA1 and LHY are MYB-like transcription factors (Schaffer et al., 1998; Wang and Tobin, 1998). Most MYB proteins have a MYB domain region that binds to a DNA sequence in the promoter region of a gene in a sequence-specific manner , and a C-terminal region which contains activation domains giving the majority of MYB factors transcriptional activation function (Jin and Martin, 1999; Ito, 2005). mRNA and protein abundance of *CCA1* and *LHY* cycle in circadian manner, i.e. oscillation persists when plants are moved from 12:12 L:D to continuous light (Schaffer et al., 1998; Wang and Tobin, 1998). Plants with loss-of-function of *CCA1* or *LHY* have a shortening of circadian period in expression of clock controlled genes as well as phenotypic outputs such as leaf movement (Green and Tobin, 1999; Alabadi et al., 2002). In addition, both mutants flower earlier in comparison to the wild type (Mizoguchi et al., 2002). In contrast, overexpression of either *CCA1* (*CCA1*-OX) or *LHY* (*LHY*-OX), driven by the *CaMV* 35S promoter, causes hypocotyl elongation and delays flowering in *Arabidopsis* (Schaffer et al., 1998; Wang and Tobin, 1998; Green and Tobin, 2002). Overexpression also disrupts rhythmicity of circadian regulated genes (e.g. *CAB*, *CCR2*, etc.) when under continuous light or dark conditions (Schaffer et al., 1998; Wang and Tobin, 1998). *CCA1*-OX and *LHY*-OX phenotypic association with disruption of the circadian clock indicates that rhythmic expression of *CCA1* and *LHY* drives overall circadian rhythmicity (Schaffer et al., 1998; Wang and Tobin, 1998; Carre and Kim, 2002).

Even though *CCA1* and *LHY* have been accepted as components of the main loop of the *Arabidopsis* circadian clock, inactivation of both genes does not result in

a complete loss of circadian rhythmicity (Mizoguchi et al., 2002; Lu et al., 2009). Indeed, *cca1-11:lhy-21* double knockout has a profound effect on *Arabodpsis* phenotype, causing early flowering and shorter hypocotyl length than the wild type or either single mutant. However, circadian rhythmicity of clock controlled genes is not abolished and persists with a period reduced to 19 h (Mizoguchi et al., 2002; Ding et al., 2007; Niwa et al., 2007; Lu et al., 2009). This data supports the importance of CCA1 and LHY in maintaining a normal 24 h circadian period, although if both are absent, other proteins or interlocking feedback loops, such as GI-TOC1, could partially compensate (Locke et al., 2006; Lu et al., 2009).

Similarities in *cca1-* and *lhy-* null phenotypes suggest that CCA1 and LHY are functionally redundant and can substitute for one another (Alabadi et al., 2002; Carre and Kim, 2002; Locke et al., 2005a). Indeed, in *Arabidopsis*, CCA1 and LHY share an overall amino acid identity of 42%, with an almost identical MYB domain region and 5 other regions of highly similar sequence (Figure 1.5) (Schaffer et al., 1998; Carre and Kim, 2002; Oda et al., 2007). The C-terminal sequences of CCA1 and LHY are less conserved, but this is a common feature amongst MYB family proteins (Schaffer et al., 1998; Jin and Martin, 1999). Both CCA1 and LHY have been found to transcriptionally regulate the same set of midday- and evening-specific genes by binding to the same promoter fragments (Wang et al., 1997; Harmer et al., 2000; Spensley et al., 2009). For example, Wang et al. (1997) showed that CCA1 interacts with two adjacent fragments of the *Lhcb1*3* promoter and that the MYB domain of CCA1 is required for this interaction. It has been recently demonstrated that LHY also binds to the same fragment of the Lhcb1*3 promoter (Lu et al., 2009).

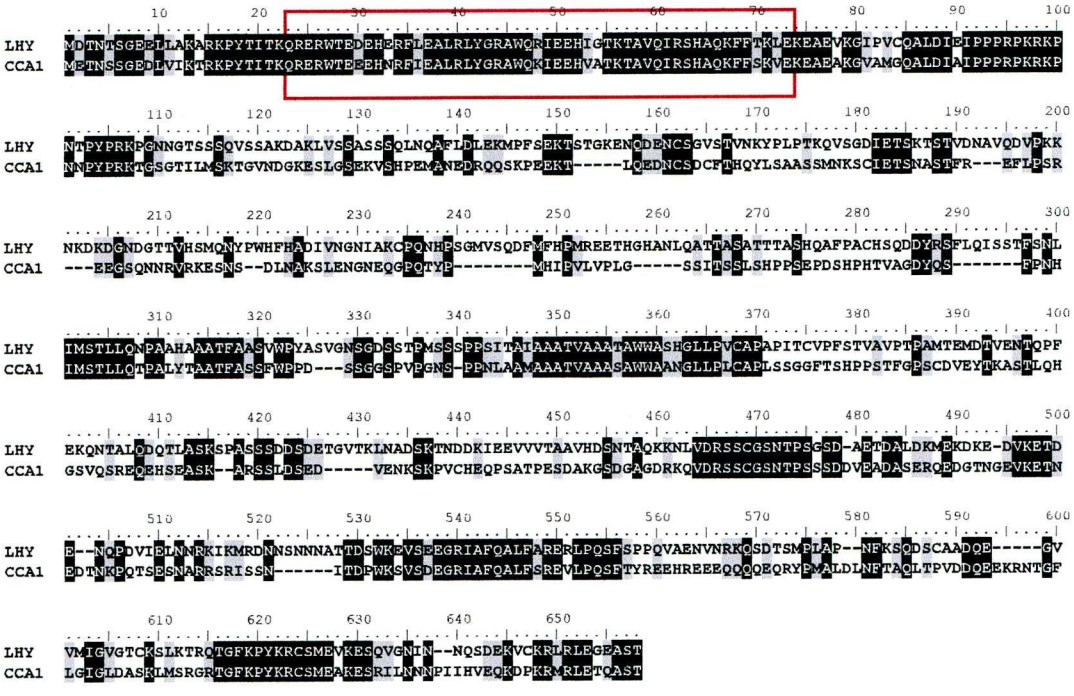


Figure 1.5. ClustalW alignment of CCA1 and LHY protein sequences.

Sequences were obtained from The Arabidopsis Information Resource (TAIR) website, www.arabidopsis.org. The red box encompasses the MYB domain.

Another sequence, frequently found in promoters of circadian regulated genes, is the evening element (EE) (Harmer et al., 2000; Harmer and Kay, 2005). The EE is present in the promoter of *TOC1* and is targeted by CCA1 and LHY, resulting in the inhibition of *TOC1* expression (Alabadi et al., 2001). However, not all EE containing genes are negatively regulated by these MYB proteins. For example, the *CCR2* (*COLD CIRCADIAN RHYTM RNA BINDING*) promoter also contains an EE and has been shown to be regulated by CCA1 and LHY (Harmer and Kay, 2005). Although, even though expression of *CCR2* is highly suppressed in mutants overexpressing *CCA1* or *LHY*, the expression is also reduced in mutants lacking *CCA1* and *LHY*, suggesting a combined involvement of activators and suppressors in generating a rhythm (Kim et al., 2003; Harmer and Kay, 2005).

CCA1 and LHY are not only functionally redundant, but also share similar regulatory mechanisms, e.g. transcriptional induction of *CCA1* and *LHY* by light. The transcription factor *PIF3* (*PHYTOCHROME INTERACTING FACTOR*) binds to G-box promoter elements, found on *CCA1* and *LHY* promoters, and mediates their interaction with phytochrome molecules, which act as light sensors (Ni et al., 1998; Martinez-Garcia et al., 2000; Michael and McClung, 2003). Light mediated induction has been confirmed by exposing etiolated *Arabidopsis* seedlings to light and measuring the light-induced increase in the transcript levels (Wang and Tobin, 1998; Martinez-Garcia et al., 2000).

It has already been mentioned that circadian rhythmicity in *CCA1*-OX and *LHY*-OX in LL or DD conditions is abolished (Wang and Tobin, 1998; Schaffer et al., 1998). Interestingly, rhythmic expression of the circadian clock-controlled genes, as well as endogenous *CCA1* and *LHY*, is present in these overexpressors when plants are grown under diurnal (light:dark) conditions (Green et al., 2002; Kim

et al., 2003). Furthermore, in *LHY*-OX plants expression of the luciferase reporter gene, driven by either the *CCA1* or *LHY* promoter, exhibits diurnal oscillation (Kim et al., 2003). All rhythms are abolished on the transfer of *CCA1*-OX and *LHY*-OX to LL or DD, indicating that the ability to anticipate dawn/dusk has been lost, however the responsiveness to light induction is retained (Green et al., 2002; Kim et al., 2003).

It has also been noted that *CCA1* and *LHY* can regulate their own and each other's transcription. In *CCA1*-OX, under LL conditions, expression of endogenous *CCA1* and *LHY* is highly suppressed and rhythmicity is abolished (Wang and Tobin, 1998; Kim et al., 2003). This has also been supported by a *CCA1* promoter-*GUS* reporter gene assay. Analysis of its activity in *CCA1*-OX plants revealed that *GUS* expression was suppressed (Wang and Tobin, 1998). Intriguingly, as in *CCA1*-OX, native *LHY* transcript rhythmicity is abolished in *LHY*-OX of but, unlike *CCA1*, it was noticed that *LHY* expression was not completely reppressed (Schaffer et al., 1998). These results suggested that transcriptional regulation of *CCA1* and *LHY* could be mediated through slightly different mechanisms. Indeed, it is quite possible that *CCA1* and *LHY* are not entirely analogous in all respects. One of the examples is the difference between *CCA1* and *LHY* mRNA degradation rates. It has been calculated that the half-life of a *CCA1* transcript, measured in light grown *Arabidopsis* treated with the transcription inhibitor cordycepin, is 1.5h, whilst *LHY* mRNA half-life is more than 2.1 h (Lidder et al., 2005; Yakir et al., 2007). Interestingly, the stability of *CCA1* mRNA is not constant throughout the day, being more stable at night and degrading faster upon exposure to light (Yakir et al., 2007). By contrast, *LHY* has been shown to be light sensitive on the translational level (Kim

et al., 2003). *LHY* protein synthesis is highly light inducible, however this process is gated and is under circadian control (Kim et al., 2003).

It has also been shown that phosphorylation of CCA1 by the protein kinase CK2 is important in its circadian function (Sugano et al., 1998). CK2 can phosphorylate CCA1 and LHY (Sugano et al., 1999). Phosphorylation by CK2 is important in CCA1-DNA complex formation as well as protein-protein interaction, for example formation of the CCA1/CCA1 homodimer (Daniel et al., 2004). New studies have emerged demonstrating the significance of interaction between CCA1 and LHY proteins, i.e. formation of homo and hetero dimers, in ensuring proper circadian function (Lu et al., 2009; Yakir et al., 2009). Lu et al. (2009) has revealed that CCA1 interacts with LHY via a region outside the DNA-binding MYB domain. Keeping in mind that 2 MYB domains are commonly required for proper DNA binding, it is possible that heterodimerization is essential for the role of CCA1 and LHY as transcription factors and in binding DNA efficiently (Yakir et al., 2009). It is, however, not known whether homodimerization of CCA1 and LHY is of the same importance as the CCA1-LHY complex or whether this process is induced by some environmental stimuli. It also hasn't been determined whether formation of homodimers versus heterodimers results in different biochemical properties, thus influencing the circadian oscillator. Such variation could explain the different roles *CCA1* and *LHY* play in the temperature compensation mechanism of the *Arabidopsis* circadian clock (Gould et al., 2006).

1.7. Temperature compensation in *Arabidopsis*

As previously stated, circadian clocks are temperature compensated and circadian rhythms persist over a broad range of temperatures. The temperature compensation mechanism has been well dissected in *Neurospora.* However, data on

the mechanism in *Arabidopsis* is only starting to emerge. The gene *GI* has no apparent effect at 17°C, but seems to be important in maintaining 24 h rhythmicity if temperature deviates from this, i.e. plants lacking GI display a period shortening at 12°C, 22°C and 27°C, but has no effect at the normal temperature (17°C) (Park et al., 1999; Gould et al., 2006). *GI* is involved in regulation of *CCA1* and *LHY* (Fowler et al., 1999), and studies of *GI* by Gould et al. (2006) have revealed that this regulation is temperature-dependant. At 17°C, no difference in *CCA1* levels is observed between the wild type control and the *gi*-null plants, however, in the mutant the *CCA1* expression becomes almost completely repressed at 27°C. By contrast, *LHY* expression is down-regulated at all examined temperatures (12°C, 17°C and 27°C), suggesting a different mechanism of interaction between *GI* and *LHY* versus *GI* and *CCA1*. Furthermore, in the wild type levels of *CCA1* and *LHY* also change in response to temperature (Figure 1.6, from Gould et al., 2006).

Differentiation between the roles of *CCA1* and *LHY* in temperature compensation has also been supported by analysing expression of a *CAB::LUC* reporter gene in plants lacking functional CCA1 or LHY. At 27°C, the *CAB::LUC* circadian period in the *lhy*-null mutant was 2 h shorter than in the wild type, whilst in the *cca1* mutant it differed by less than 1 h. By contrast, at a low temperature (12°C), circadian rhythm of the *cca1*-null was more affected (Figure 1.7, from Gould et al., 2006). This indicates that functioning *LHY* is of more significance in buffering the clock at high temperatures, when *CCA1* is more important at low temperatures. Overall, it is apparent that both these genes contribute to the proper functioning of the circadian oscillator and their functional redundancy is only partial, at least when exposed to different temperatures.

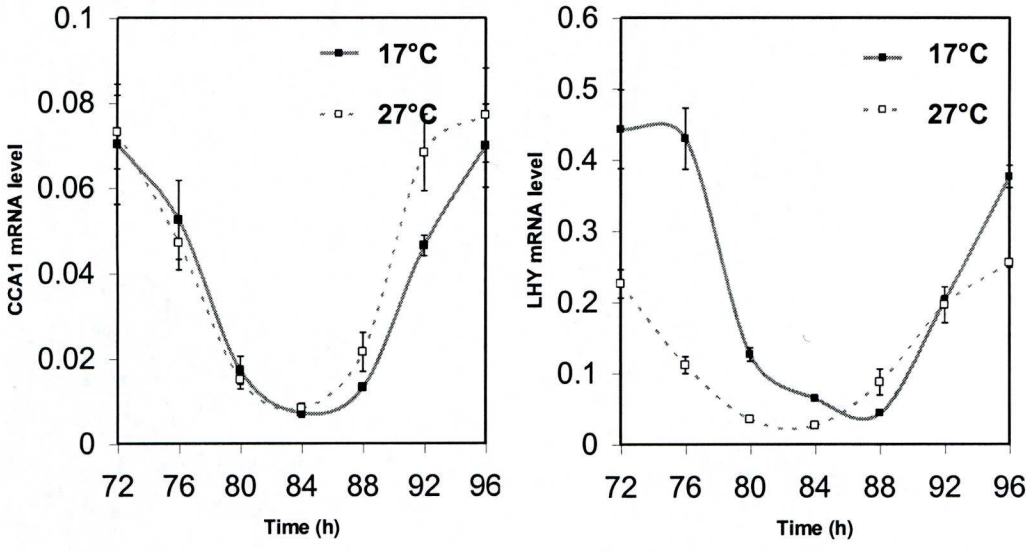


Figure 1.6. *CCA1* and *LHY* mRNA expression at different temperatures (from Gould et al., 2006).

Seedlings were grown at 22°C under 12:12 L:D photoperiod for 7 d and then moved to constant light conditions at 17°C or 27°C. Plants were harvested after 72 h in constant conditions and every 4 hours for the next 24 h. Error bars represent SE.

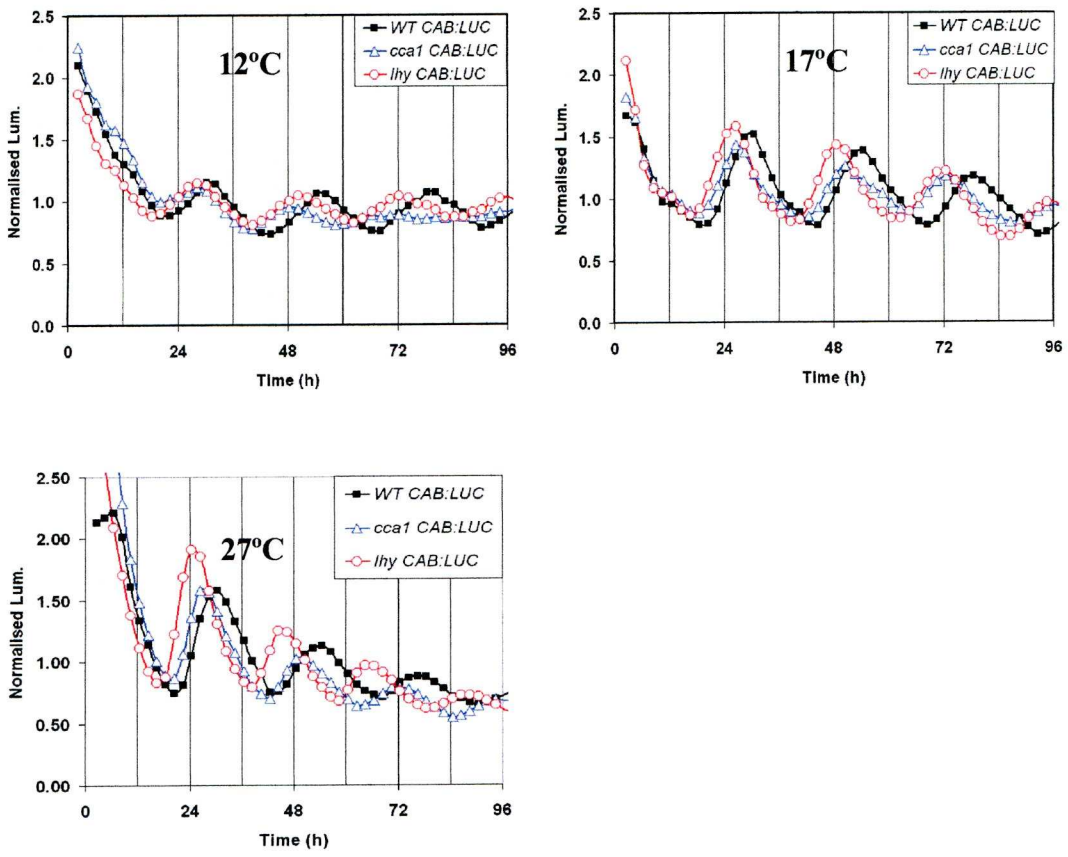


Figure 1.7. Expression of *CAB:LUC* in wild type (WT), *cca1* and *lhy* mutants at 12°C, 17°C and 27°C (Gould et al., 2006).

Seedlings were grown at 22°C under 12:12 L:D photoperiod for 7 d and then moved to constant light conditions at 12°C, 17°C or 27°C, where luminescence was monitored.

1.8. AIM of PhD

This project's aim was to gain more knowledge about temperature compensation in the *Arabidopsis* circadian clock with an emphasis on *CCA1* and *LHY*, which encode two MYB transcription factors that function at the core of the oscillator. Several questions are addressed in this thesis:

1) Is there a difference between the promoter-mediated regulation of *CCA1* and *LHY*, and does this regulation depend on temperature? If the difference in temperature dependent regulation between *CCA* and *LHY* does exist, can it account for the different roles CCA1 and LHY have in temperature compensation? These questions were addressed by switching the *CCA1* and *LHY* promoters between the two genes, and analyzing the effects of these new chimeras on the circadian clock and temperature compensation.

2) The effect of the increased dosage of *CCA1* and *LHY* on the circadian clock and its temperature compensation will be investigated. This was achieved by creating transgenic plants carrying extra copies of *CCA1* and *LHY*.

3) Is there natural variation in temperature compensation amongst different circadian clock outputs and are all of the outputs temperature compensated? Whilst existence of natural variation in temperature compensation has been reported previously, it is not clear whether all circadian clock parameters are buffered against temperature differences to the same degree. Here, temperature compensation was compared between different geographical accessions of *Arabidopsis* by using leaf movement as well as *CCA1* and *LHY* bioluminescence assays. Effect of the temperature compensated clock on *Arabidopsis* growth performance was also investigated.

Chapter 2 - Materials and Methods

2.1. Plant material:

2.1.1. Plant material used for analysis of *CCA1* and *LHY* promoters:

2.1.1.1. Non-transgenic plant material

Arabidopsis thaliana L. Heynh ecotype Wassileskija (Ws-2, but Ws thereafter) was obtained from A. Hall, which had been originally purchased from NASC (The European Arabidopsis Stock Centre in Nottingham) (seed NASC ID N1601). *cca1-11* and *lhy-21* null in a Ws background have been previously described in Hall et al. (2003). *cca1-11* carries a T-DNA insertion 80 bp upstream from the ATG start codon and *lhy-21* 100 bp after the start of the seventh exon.

2.1.1.2. Construction of CCA1 and LHY transgenic plants

2.1.1.2.1. Fusion PCR technique

Fusion PCR has been used as a rapid way of replacing different parts of genes or gene tagging with avoidance of time consuming cloning procedures (Szewczyk et al., 2007). During the first set of PCR reactions, DNA fragments to be fused, are separately amplified using specifically designed primers. In Figure 2.1, the red fragment is amplified with primers P1 and P2 and the blue fragment with primers P3 and P4. One part of the primer P2 is complementary to the section of the red DNA while the other part of P2 is complementary to the section of the blue DNA. On the other hand, primer P3 has a part complementary to the end of the red DNA and a part complementary to the blue DNA. Such design of primers ensures production of PCR fragments with flanking "tails" complementary to each other – the basis for fusion of PCR products.

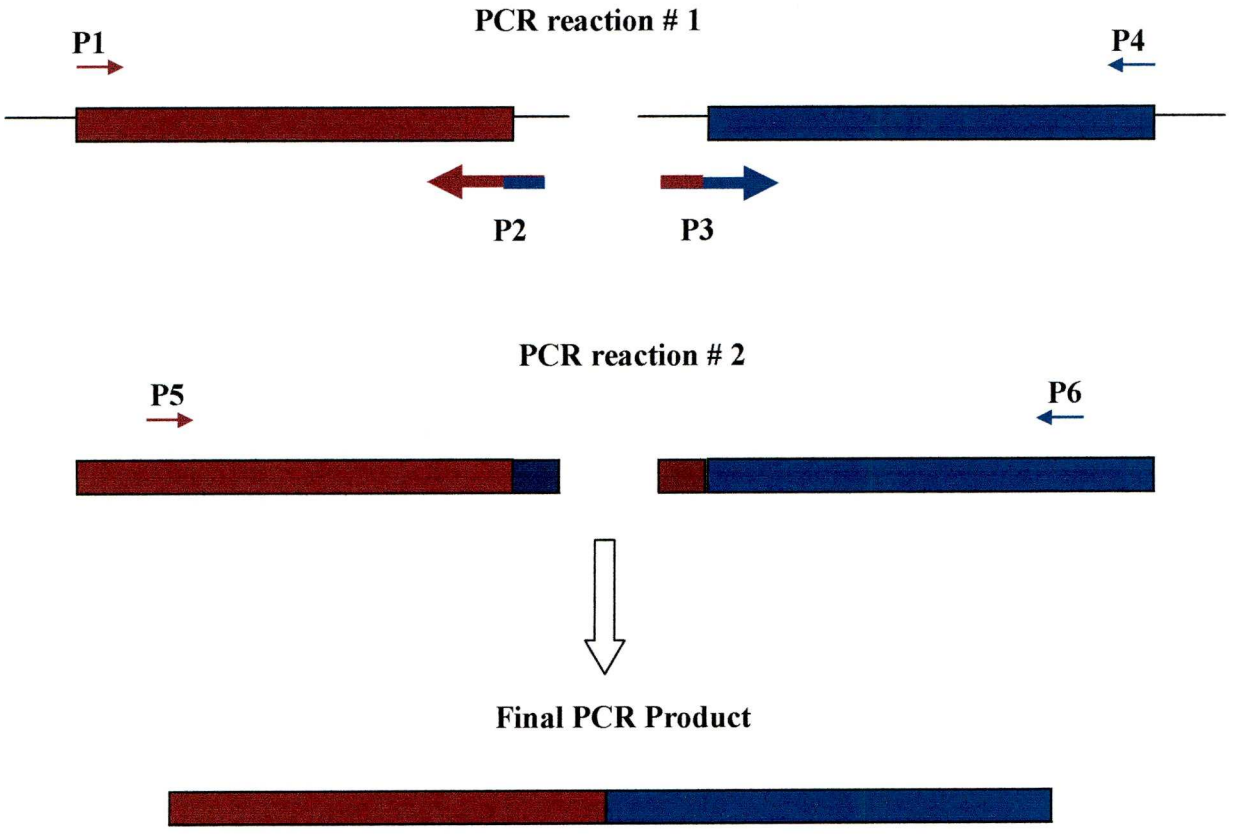


Figure 2.1. A schematic diagram of primers designed to fuse two DNA fragments (red with blue).

DNA fragments are first amplified with primers P1 and P2 and with P3 and P4. Primers P2 and P4 are designed to produce fragments containing "tails" that are complementary to the part of the DNA to be fused with. Two DNA fragments are mixed and fused by PCR using nested primers P5 and P6.

During the second round of PCR, fragments amplified during the first PCR reaction set, are mixed together and fused using "nested " primers, corresponding to P5 and P6 on Figure 2.1. Flanking "tails" will anneal to each other creating a new fusion product. Fusion PCR technique proved to be an efficient and fast method for making chimeric constructs.

2.1.1.2.2. DNA isolation and preparation of the fusion constructs/chimeras

Genomic DNA from *Arabidopsis* seedlings was extracted using Plant DNAzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The oligonucleotide primers for the fusion PCR were designed based on the strategy described in the previous section (Figures 2.1) and are presented in Table 2.1 and Figures 2.2 A and 2.2 B)

The substitution of promoters to create *CCA1::LHY* and *LHY::CCA1* constructs was achieved by two-step PCR. During the first step the *CCA1* promoter, CCA1 protein coding region (ORF), *LHY* promoter, and *LHY* ORF were separately amplified using primer pairs CCA1-LP and CCA1-RPF, CCA1-LGF and CCA1-RG, LHY-LP and LHY-RPF, and LHY-LGF and LHY-RG respectively. Amplified PCR products were excised from a 2% (w/v) agarose gel and purified using MinElute gel extraction kit (Qiagen, Hilden, Germany). During the second round of PCR, the *CCA1* promoter was fused to the *LHY* ORF using nested primers CCA1 – LPN and LHY – RGN, and the *LHY* promoter fused to the *CCA1* ORF using primers LHY – LPN and CCA1 – RGN (Table 2.1). Constructs *CCA1::CCA1* and *LHY::LHY* were created using nested primers CCA1 – LPN and CCA1 – RGN, and LHY – LPN and LHY – RGN respectively.

Table 2.1. List of primers used for the fusion PCR

Name	Primer sequence (5' to 3')	DNA template used	Generated PCR product
	First round		
CCA1 – LP	5'-cctttctctctctgtctctggtctt-3'	Genomic DNA	*CCA1* promoter
CCA1 – RPF	5'-gtattagtatccatcactaagctcctctacacaacttc-3'		
CCA1 – LGF	5'-gtcctgttatggagacaaattcgtctggagaagatc-3'	Genomic DNA	*CCA1* ORF
CCA1 – RG	5'-cagatgattatagcaagctgaaaaagag-3'		
LHY – LP	5'-tttataattccaaacatcaacgtagag-3'	Genomic DNA	*LHY* promoter
LHY – RPF	5'-cgaatttgtctccataacaggaccggtgcagc-3'		
LHY – LGF	5'-gcttagtgatggatactaatacatctggagaagaattattagc-3'	Genomic DNA	*LHY* ORF
LHY – RG	5'-gttggtctagcaagtgattgtaaaatgt-3'		
CCA1 – LPN	5'-gtcaaagtgttgtaaattcctcaagact-3'	Genomic DNA	*CCA1::CCA1* construct
CCA1 – RGN	5'- caactctgttgtacttgctactgcttg -3'		
LHY – LPN	5'-tttataattccaaacatcaacgtagag-3'	Genomic DNA	*LHY::LHY* construct
LHY – RGN	5'- attatcgtaacactcactacactaccactc-3'		
	Second round		
CCA1 – LPN	5'-gtcaaagtgttgtaaattcctcaagact-3'	*CCA1* promoter	*CCA1::LHY* construct
LHY – RGN	5'-attatcgtaacactcactacactaccactc-3'	*LHY* ORF	
LHY – LPN	5'-tttataattccaaacatcaacgtagag-3'	*LHY* promoter	*LHY::CCA1* construct
CCA1 – RGN	5'-caactctgttgtacttgctactgcttg-3'	*CCA1* ORF	

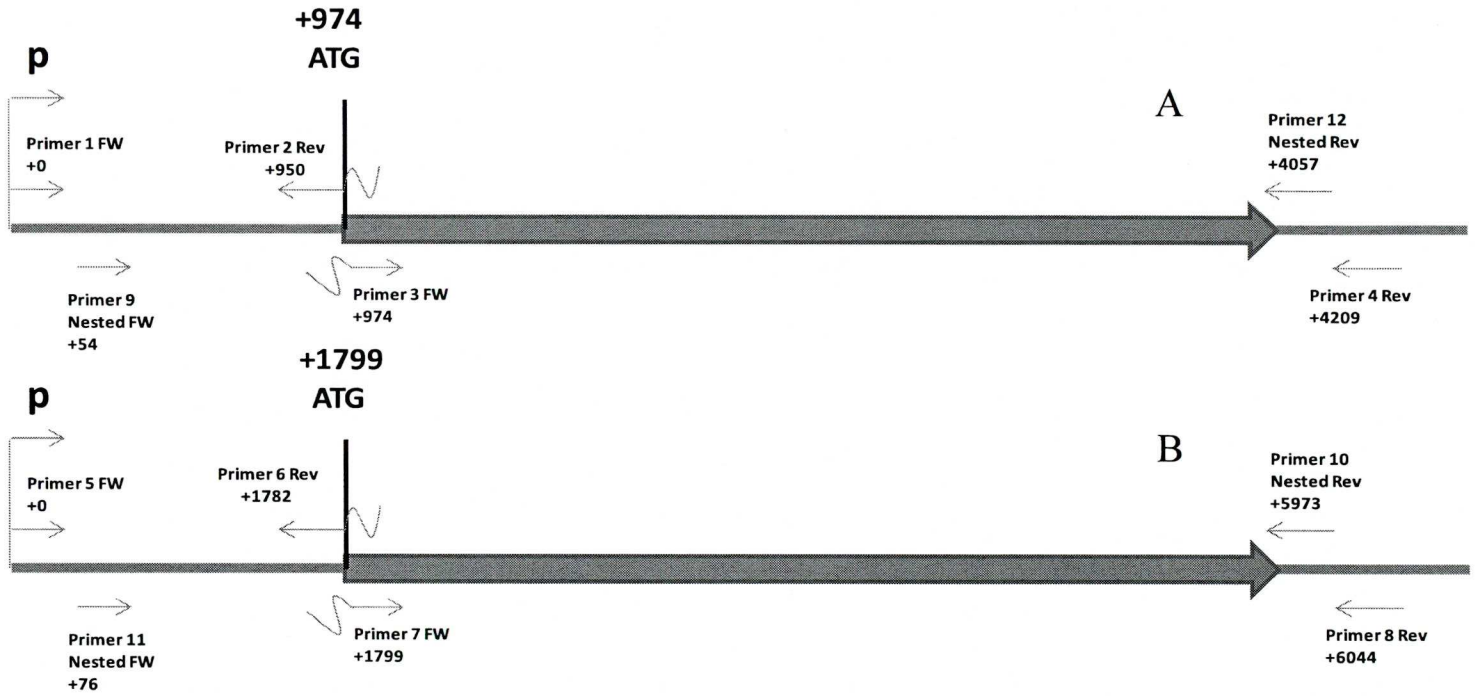


Figure 2.2. A schematic representation of the *CCA1* (A) and *LHY* (B) genes and their promoters, including the annealing positions of the primers used in fusion PCR.

p – promoter, position +0 – first nucleotide of primer 1. Primer pairs 1FW with 2Rev and 5FW with 6Rev amplify the promoter region and pairs 3FW with 4Rev and 7FW with 8Rev amplify the coding region of the gene. FW – forward, Rev – reverse. Primers 2, 3, 6 and 7 consist of 2 parts, where one part is complementary to the target sequence (straight line). The second part is the flanking "tail" which is used to fuse PCR products during the second round PCR (wavy line). The final product is amplified with nested primers 9, 10, 11 and 12. Primers' numbers correspond to their numbers in Table 2.1.

All PCR reactions were carried out in a mixture containing 2.5 µl 10x PCR buffer for KOD DNA polymerase, 2 mM $MgSO_4$, 0.2 mM of each dNTP, 0.5 U of KOD Hot Start DNA polymerase (proofreading high-fidelity DNA polymerase) (Novagen), 10-30 ng of DNA template and sterile water to bring the total volume to 25 µl. PCR was performed in a Dyad DNA Engine Peltier thermal cycler (MJ Research). Hot start PCR was initiated with 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 57°C for 30 s, and 72°C for 2-5 min (depending upon product size) with a final elongation step at 72°C for 7 min.

2.1.1.2.3. Plasmid transformation

Constructs *CCA1::CCA1*, *CCA1::LHY*, *LHY::LHY* and *LHY:CCA1* were cloned into GATEWAY pCR®8/GW/TOPO entry vector (Appendix 1) using pCR®8/GW/TOPO® TA cloning® kit (Invitrogen) following the manufacturer's instructions. One Shot® chemically competent *Escherichia coli* were used for transformations. Transformed cells were selected on LB (Lysogeny broth) agar plates with 100 µg/ml spectinomycin.

Introduction of constructs into *E. coli* was confirmed by colony PCR, which involved picking 6-10 random colonies and transferring them directly into the PCR mixture using a pipette tip. The PCR mixture contained the same ingredients for the KOD PCR described in the previous section. Plasmid DNA was isolated from the confirmed colonies using QIAprep® miniprep kit (Qiagen). It was then analyzed by *Eco RI* restriction enzyme digestion. The correct orientation of the introduced construct was confirmed by sequencing with M13 Forward and M13 Reverse primers. Once correct transformants were identified, the whole product was sent off for full sequencing. Sequencing primers were designed at 400 bp intervals by reference to the full *Arabidopsis* genome sequence obtained from The Arabidopsis

Information Resource (TAIR) (www.arabidopsis.org). Relatively short intervals were chosen to ensure good coverage of the sequence.

Once the sequences of all chimeric genes were confirmed, entry vectors were recombined with the pMDC100 Gateway destination vector (Appendix 2) by recombinase reaction using Gateway™ LR Clonase™ Plus enzyme mix (Invitrogen) (Curtis and Grossniklaus, 2003). pMDC100 contains a pCambia 2300 backbone (www.cambia.org) with a bacterial kanamycin resistance marker, to select transformed *E. coli* and *Agrobacterium tumefaciens,* and a plant kanamycin resistance marker to select transformed plants. For LR recombination, a concentration of 200 ng/µl DNA was used for both vectors. After incubating mixtures at 25°C for 16 h, reactions were stopped by adding protenaise K (1 µl for a 10 µl reaction) and were incubated at 37°C for an additional 10 min. LR reaction mixtures were transformed into One Shot® chemically competent *E. coli* and plated out on LB agar with 50 µg/ml kanamycin. Kanamycin resistant transformants were also analyzed by colony PCR using nested primers) (Table 2.1) to confirm the presence of chimeras. Subsequently isolated plasmid DNA was also analyzed by *Eco* RI restriction enzyme digestion.

2.1.1.2.4. Transformation of Agrobacterium

Agobacterium tumefaciens strain GV3101 pMP90 was transformed with the pMDC100 vectors by a freeze-thaw method, which involves freezing vials containing 400 ng/µl of vector per 100 ul of *Agrobacterium* in liquid nitrogen before thawing them at 37°C for 5 min (An et al., 1988). After addition of 1 ml of room temperature YEBs (Bacto Yeast Extract) (5g Bacto Beef Extract [Difco], 5 g Bacto-Peptone [Difco], 1 g Bacto Yeast Extract [Difco, Detroit, USA], 0.5 g $MgS0_4x7H_2O$, and 5 g sucrose per litre [pH 7.2 with NaOH]), the culture was allowed to recover at

28°C with shaking in an Incubator Shaker (New Brunswick Co. Inc., Innova™ 4300) for 3 h at 150 rpm. Recovered cells were centrifuged at 13 000 rpm for 30 s (Eppendorf Centrifuge 5415R) and resuspended in 100 µl of fresh YEBs. Resuspended cells were spread onto 1.5% agar YEBs plates containing 50 µg/ml kanamycin, 50 µg/ml gentamicin and 50 µg/ml rifampicin. Plates were incubated at 28°C for 3 days. Presence of chimeras was confirmed by colony PCR and an *Eco* RI restriction enzyme digestion of plasmid DNA isolated from resistant *Agrobacterium* colonies.

2.1.1.2.5. Plant transformation

Arabidopsis plants were transformed with *A. tumefaciens* via the floral dip method (Clough and Bent, 1998; Davis et al., 2009). For this, selected *A. tumefaciens* colonies transformed with the appropriate constructs were cultured in 10 ml of YEBs plus 50 µg/ml kanamycin, 50 µg/ml gentamicin, 50 µg/ml rifampicin on a 150 rpm shaker at 28°C. After 24 h, 10 ml of the culture was resuspended in 500 ml of fresh YEBs and incubated for an additional 24 h. For dipping plants, *A.tumefaciens* culture was poured into a beaker with addition of 0.02% Silwet L-77 (Lehle seeds, USA). Floral parts of *Arabidopsis* were subsequently dipped into the beaker with the inoculum and left for 10 s. Inoculated plants were placed in separate plastic bags and sealed to maintain humidity. The bags were opened the next day to avoid rotting of plants. Transformed plants were grown in the greenhouse and seeds were harvested upon maturation. Seed of transformed *Arabidopsis* from each pot was combined together.

2.1.1.2.6. Selection of transformed, homozygous for the insert, plants

Surface sterilized seeds from the primary transformants (T_0) were sown on 1.5% agar plates containing 50 µg/ml kanamycin as successfully transformed

Arabidopsis should carry the kanamycin-resistance selectable marker gene. After 7 days, plates were checked to select transformed seedlings. Kanamycin-resistant ($Kan^{(r)}$) seedlings appear green and have a long radicle. Non-kanamycin resistant ($Kan^{(s)}$) seedlings are yellow and have very short roots. Approximately 10 successful transformants were transferred into soil and left to set seed – T_1 (first transformed generation seed).

A. tumefaciens usually only targets female reproductive tissue (Desfeux et al., 2000), thus first generation transformants should be heterozygous. Transformed genes are commonly transmitted according to the Mendelian segregation rule, therefore T1 seed with one functional insert should result in 3 $Kan^{(r)}$:1 $Kan^{(s)}$ (kanamycin resistant:kanamycin sensitive) segregation ratio (Feldmann, 1991). As the kanamycin resistance gene is a dominant marker, $Kan^{(r)}$ seedlings encompass homozygous and heterozygous transformants, while non-resistant are untransformed individuals (Feldmann et al., 1997). Following these assumptions, only progeny from plant lines segregating at approximately 3:1 $Kan^{(r)}$:$Kan^{(s)}$ were selected for further screening. Approximately 10 transformants were transferred into soil to set seed – T_2 (second transformed generation seed). T_2 seed was yet again screened on kanamycin and only 100% resistant plant lines were chosen to set seed (T_3), indicating that the parents were homozygous for the insert. Homozygous T_3 lines were used for circadian function analysis.

2.1.2. Plant material used for the analysis of natural variation

2.1.2.1. Geographical Arabidopsis accessions

Seed from 13 *Arabidopsis* accessions was obtained from A. Hall's lab. Additional accessions Je54 and Or-0 had been previously identified as heat sensitive and Wc-1, Dog-5 and Phw-19 as heat tolerant by J.Burke (pers. commun.) and were

also included in this study. Seed for these accessions was purchased from TAIR (www.arabidopsis.org). Information on geographical location for all *Arabidopsis* accessions from this study is presented in Table 2.2.

2.1.2.1. Generation of CCA1:LUC and LHY:LUC transgenic plants

2.1.2.1.1. Plant transformation

The *CCA1:LUC* and *LHY:LUC* transgenes, separately subcloned into the plant pPCV812 binary vector, were obtained from A. Millar (Locke et al., 2005). This vector contains ampicillin bacteria-selectable marker gene and hygromycin plant-selectable marker gene. pPCV vectors, carrying *CCA1:LUC* or *LHY:LUC,* were introduced into *A. tumefaciens* strain GV3101 via the freeze thaw method described in section 2.1.1.2.4. *A. tumefaciens* was used to transform *Arabidopsis* accessions listed in Table 2.1 via the floral dip method (Clough and Bent, 1998; Davis et al., 2009) as described in section 2.1.1.2.5. Transformed plants were left to recover and senesce. Seeds from dry plants were harvested on maturity.

2.1.2.1.2. Selection of transformed, homozygous for the insert, plants

Surface sterilize seeds from the primary transformants were screened on 1.5% agar containing 50 µg/ml hygromycin as decribed in section 2.1.1.2.6. Hygromycin-resistant seedlings appeared dark green and had longer hypocotyls in comparison to non-hygromycin resistant seedlings. Second generation seeds that segregated according to the Mendelian segregation rule were selected as described in 2.1.1.2.6. Seed of the second generation 100% hygromycin resistant plants that strongly luminesce were used in a bioluminescence assay to monitor *CCA1* and *LHY* promoter activity. Four representative transgenic lines were analyzed for each construct for each accession per experiment.

Table 2.2. Geographical information for the 18 accessions used in this study.

Information of collection sites was obtained from TAIR (www.arabidopsis.org). n/a – information not available.

	Geographical location			
Name	Country	Latitude (°)	Longitude (°)	Altitude (m)
An-1	Belgium	N 51-52	E 4-5	1-100
C24	Portugal	n/a	n/a	n/a
Col-0	Germany	N 50	E 8	1-100
Ct-1	Italy	N 37-38	E 15	1-100
Cvi	Cape Verde Island	N 15-17	W 23-25	1200
Dog-5	Turkey	N 38.3	E 42	1503
Eri	Sweden	N 56.4	E 15.4	n/a
Est	Estonia	N 59	E 26	100-200
Fei-0	Portugal	N 40	W 8	100-300
Je-54	Former Czechoslovakia	N 50	E 15	n/a
Kyo	Japan	N 35.3	E 135.9	n/a
Ler	Germany	N 53	E 15-16	1-100
Or-0	Germany	N 50.5	E 7.6	n/a
Phw-19	UK	N 51.2	E 0.9	n/a
Sha	Tadjikistan	N 39	E 70	3400
Van-0	Canada	N 49-50	W 123	1-100
Wc-1	Germany	N 53	E 10	1-100
Ws	Belarus	N 52	E 30	100-200

2.2. Plant growth conditions

2.2.1. Seed sterilization

2.2.1.1. Surface sterilization

Seeds were surface sterilized in 70% ethanol for 1 min, 50% bleach with 0.01% Tween 20 for 10 min followed by one rinse in sterile water as in Gould et al., 2006. Sterilized seeds were then re-suspended in 0.15% agar and kept in the dark for 3 days prior to sowing.

2.2.1.2. Gas sterilization

Opened Eppendorff tubes, containing small amounts of seeds were arranged in a tray and placed in a glass desiccation jar holding 500 ml of reverse osmosis (RO) water with two dissolved chlorine tablets (CLO-TABS, Arrow Solutions) and with subsequent addition of 5 ml of hydrochloric acid. Seeds were left to sterilize for 3 h and then moved to a sterile flow hood for 1 h to allow any remaining chlorine to evaporate before being re-suspended in 0.15% agar (modified from Desfeux et al., 2000)

2.2.2. Plant growth

Sterilized seeds were sown onto 1.5% agar plates containing Murashige and Skoog media (MS). For *CCA1* and *LHY* promoter switch analysis, agar media was supplemented with 3% sucrose. Seeds were stratified in the dark at 4°C for 3 days before being moved to the 22°C room and grown under 12:12 light:dark (L:D) cycles of 80-100 $\mu mol/m^2/s$. Approximately 10 days old seedlings were used for all experiments, except for monitoring delayed fluorescence when 15 days old seedlings were used.

2.3. Characterization of circadian rhythmicity

2.3.1. Leaf movement assay

In the 18th century, the first record of circadian rhythms in plant leave movement was made (reviewed in McClung, 2006). Since then automated systems for monitoring leaf movement have been developed and are widely used as an assay for the circadian rhythmicity in different plants, including *Arabidopsis* (Dowson-Day and Millar, 1999; Edwards and Millar, 2007). The leaf movement assay involves collecting a series of images of individual seedlings taken at regular intervals. The method is easy to set up and does not require plant transformation prior to the experiment, which makes it very attractive especially for plants that are difficult to transform (Edwards and Millar, 2007).

2.3.1.1. Leaf movement system

For the leaf movement assay, surface sterilized seeds were individually sown onto agar plates. The plates were then kept in the dark at 4°C for 3 days before moving to 22°C 12:12 L:D cycles. After 10 days, agar blocks holding single *Arabidopsis* seedlings were excised from plates and arranged in rows in vertically positioned 25-compartment, 100 mm square dishes (Barloworlds Scientific, UK) (Figure 2.3).

Row position for each plant line was assigned randomly between the 16 plates used per experiment, with 2 rows representing one line. The seedlings were then moved back to the growth room for an additional day. At dawn, plates with seedlings were transferred to 2 Sanyo MLR350 growth chambers (Sanyo, Osaka, Japan) and imaged under continuous light (25 $\mu mol/m^2/s$) at either 17°C or 27°C (depending on the experiment) over the course of one week. Images were taken by

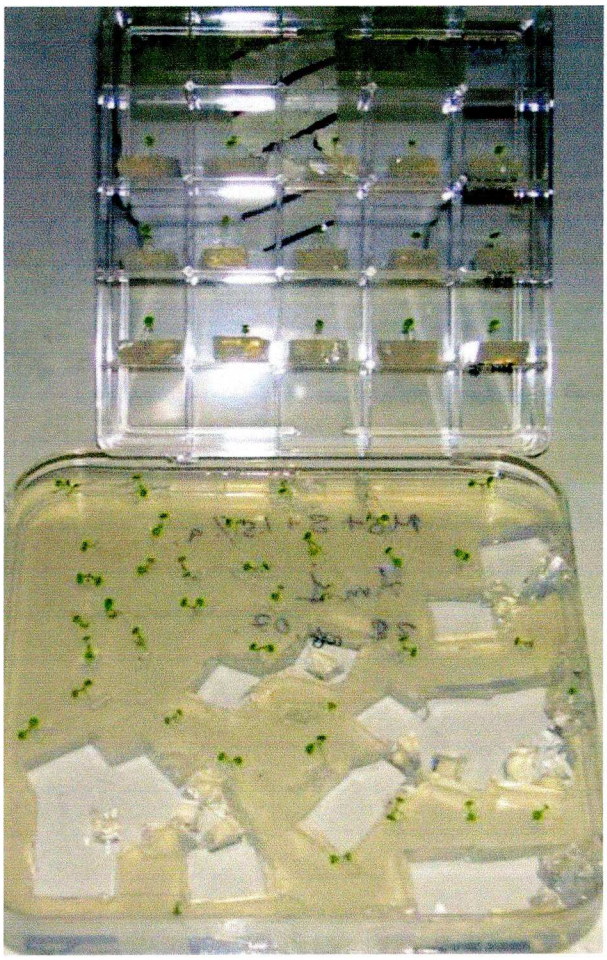


Figure 2.3. Photograph of seedling arrangement for leaf movement.

Arabidopsis seeds are individually sown onto MS agar and grown at 22°C and 12:12 L:D cycles. After 10 days agar blocks containing one seedling each are excised from plates and placed onto 25-cell dishes.

Sony Exwave HAD cameras (Soverein International) (Figure 2.4). Each growth chamber contained 8 cameras allowing simultaneous imaging of 8 plates (Figure 2.4). Metamorph 4.5 (Universal Imaging, West Chester, PA) was used to program each camera to capture images every 20 min and transfer them from the cameras to a 16-channel camera switcher and then to a computer via a Flashbus MV Pro card (Integral Technologies, Indianapolis).

2.3.1.2. Data analysis

The vertical positions of *Arabidopsis* primary leaves were analysed using Metamorph 4.5 software. A threshold was applied to all images to separate green *Arabidopsis* leaves from the background. Each leaf was then assigned an individual region with the centroid coordinates positioned in the centre of the thresholded object inside the region. The vertical and horizontal position data of centroids was logged into Microsoft Excel 2007 spreadsheets (Microsoft, CA). An 80 h window of data was analyzed by fast Fourier transform nonlinear least-squares analysis program (Plautz et al., 1997) in BRASS, a custom written Excel macro (http://www.amillar.org/downloads.html). The first 24 h were not included in the analysis to eliminate any effects of seedling adjustment to continuous light and different temperature on their transfer to the growth cabinets. Circadian period estimates and real amplitude error (RAE) were generated for individual leaves. RAE is used as a measure of rhythm robustness and ranges from 0 (a perfect fit to the cossin wave) to 1 (no fit).

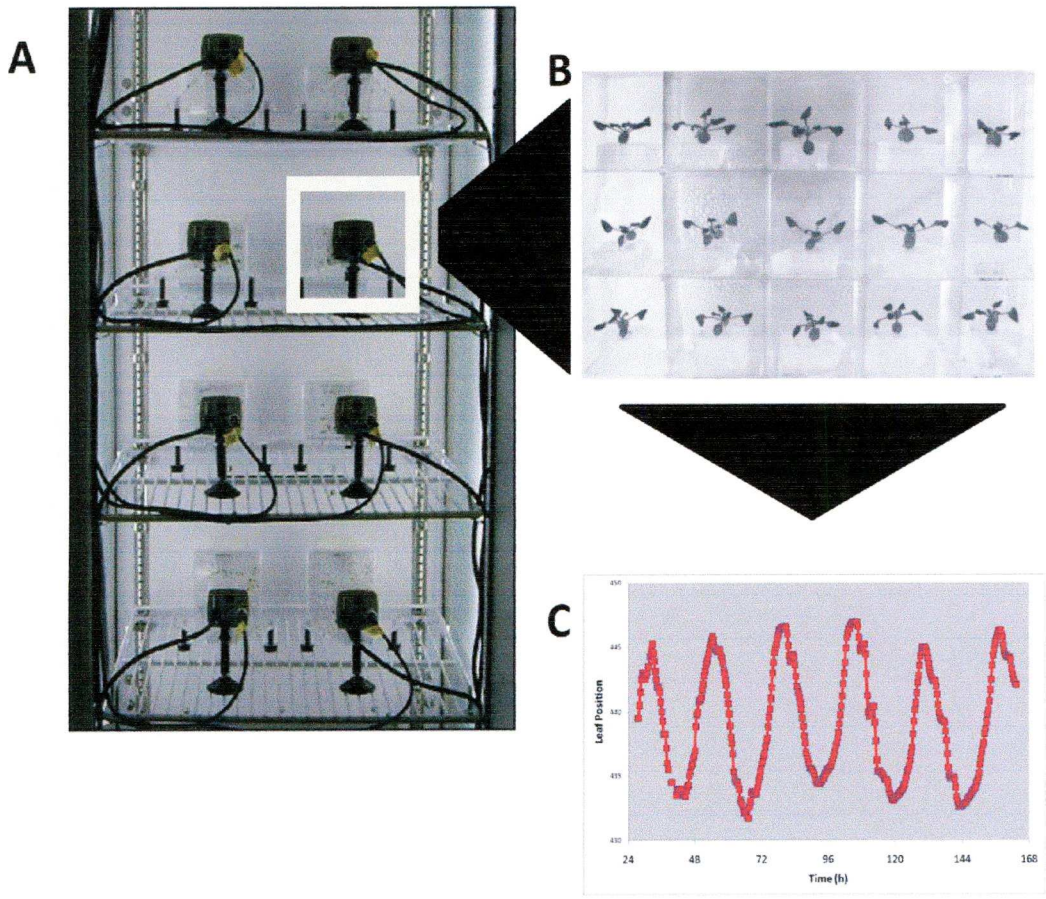


Figure 2.4. Leaf movement system

A - A photograph of a Sanyo growth chamber with 8 charge coupled device video cameras. A plate containing 10 day old *Arabidopsis* seedlings is placed in front of each camera.

B – A photograph of 25-well plate where 3 middle rows contain *Arabidopsis* seedlings. Plate images are taken every 20 minutes over the course of 5-7 days.

C – A graph representing vertical position of leaves plotted over the elapsed time. This data is obtained from analysing a series of images like B.

2.3.2. Bioluminescence assay

The bioluminescence assay is a useful method to monitor the dynamics of the gene expression. The method involves fusing a promoter of a gene of interest to the firefly luciferase (*LUC*) gene (to create reporter fusions). After luciferin application, the amount of light emitted from a transformed organism is measured, which reflects the activity of the assayed promoter. The luciferase bioluminescence assay has been proven to be an exceptional tool in monitoring gene expression and is widely used in circadian biology (Millar et al., 1995).

2.3.2.1. Bioluminescence assay setup

Gas sterilized seeds were pipetted onto 1.5 % MS agar plates in rows of 8 small clusters of approximately 20-30 seeds. When promoter switch lines were analyzed, MS agar was supplemented with 3% sucrose. A modified 1.5 ml Eppendorff tube with a removed tip was placed over each seed cluster to constrain growth of seedlings within a tube. Each row represented one *Arabidopsis* accession, carrying *CCA1:LUC* or *LHY:LUC*, and comprised four individual plant lines of 2 clusters per line. Plates with seeds were sealed and stratified at 4°C in the dark for 3 days before being transferred to 22°C and entrained to 12:12 L:D cycles of 80-100 $\mu mol/m^2/s$ for 9 days. One day before the experiment, seedlings were sprayed with filter-sterilized 5 mM D-luciferin (in 0.01% Triton X-100) in a sterile flow hood. On the day of the experiment, plates were moved to the imaging system cabinet prior to dawn so that the first light signal seedlings received was from continuous illumination (20 $\mu mol/m^2/s$ of blue light:20 $\mu mol/m^2/s$ of red light), provided by red/blue light-emitting diode (LED) arrays (MD Electronics, UK). For circadian rhythm assessment, expression of *CCA1* and *LHY* was monitored under continuous light, whilst diurnal rhythm assessment was carried out under 12:12 L:D cycles. In

this case, lights in the imaging system cabinet were controlled by a programmable timer that turned lights on and off every 12 hours. The temperature inside the cabinet was adjusted according to the experiment: 17°C, 27°C or 33°C.

2.3.2.2. Imaging system

The imaging system cabinet was a Sanyo MIR-553 incubator (Sanyo Gallenkamp, UK) with a cooled to -80°C ORCA-II-BT 1024 16-bit camera (Hamamatsu Photonics, Japan) mounted through the top of the cabinet (Southern et al., 2006). Four 12 cm x 12 cm plates of seedlings could be imaged simultaneously. Images of transgenic seedlings were taken every 2 hours. After every 1 h 35 min of illumination the light in the cabinet was switched off. After a 5 min delay, to avoid the capturing the plants' auto-fluorescence, a 20 min image was taken before the light was switched back on. This was repeated over 120 hours. Image acquisition and light inside the imaging cabinet were controlled by WASABI imaging software (Hamamatsu Photonics, Japan).

2.3.2.3. Data analysis

After collecting a series of images, image RBF files were converted into TIF files using WASABI software. Subsequent numerical analysis of bioluminescent images was performed by Metamorph 6.0 image-analysis software (Molecular Devices). Period estimates and relative amplitude errors were calculated in BRASS by running fast Fourier transformed non-linear least-square analysis (Plautz et al., 1997).

2.3.3. Delayed fluorescence assay

Delayed fluorescence is light excess from chlorophyll A, emitted after over-excitation of photosystem II by strong illumination (Rutherford et al., 1984; Gould et al., 2009). Delayed fluorescence is under circadian control and has been recently introduced as a non-invasive method of measuring circadian rhythms in plants (Gould et al., 2009). Delayed fluorescence assay uses the same camera and seed setup as bioluminescence assay.

2.3.3.1. Experiment setup and analysis

Sterilized *Arabidopsis* seed was arranged on agar plates as for the bioluminescence assay, described in 2.3.2.1. One row conferred 1 independent transformant line, or, in case of accessions, 1 not transformed accession. After 3 days of stratification seed plates were moved to a 22°C 12:12 L:D growth room for 15 days. Delayed fluorescence was measured in 15 days old seedlings using the same camera setup as for bioluminescence (described in 2.3.2.2). Images of plants were taken every hour immediately after switching lights off, using 1 min exposure. A series of images acquired over a period of 5 days were then analyzed as described in section 2.3.2.3.

2.3.4. Flowering assay

Arabidopsis seedlings were sown on soil and grown in a controlled environment under 16:8 L:D conditions at 22°C. The flowering time was expressed as a number of days until the plant had a bolt of 0.5 cm long. The number of rosette leaves was counted on that day.

2.4. RNA analysis

2.4.1. RNA extraction

Surface sterilized *Arabidopsis* was grown on MS agar plates supplemented with 3% sucrose, under the light conditions of 12:12 L:D and temperature of 22°C after 3 days of seed stratification. After 7 days of entrainment, plates were transferred to constant light at 17°C and 27°C to free-run for 72 hours. To measure *CCA1* and *LHY* RNA ratios, plant tissue was harvested at one time point, one hour after the light came on, when expression of *CCA1* and *LHY* is at maximum. For the time-course experiment, plant material was harvested every 4 h for 24 h. Approximately 100 mg of plant tissue was harvested and frozen in liquid nitrogen. Total RNA was extracted in a QIACube ® (230V) (QIAGEN Group), a robotic workstation for automated purification of nucleic acids, using QIAGEN spin-columns kits (QIAcube standard protocol: RNeasy plant mini – Plant cells and tissues – QIAshredder) and RNeasy plant mini kit (QIAGEN Group). cDNA was synthesised from 0.5 µg of template RNA using the Advantage-for-PCR retro – transcription kit (BD Bioscience).

2.4.2. Transcript analysis

Abundance of *CCA1* and *LHY* RNA was measured by quantitative PCR using Power SYBR® Green PCR master mix (Applied Biosystems, USA) in the Applied Biosystems 7500 Fast Real-Time PCR System. Transcript abundance of *CCA1* and *LHY* was normalised to a house-keeping gene *Ubiquitin* (*UBQ*). The primers for q-PCR reactions are presented in Table 2.3. All reactions were carried out in triplicate. Normalization and transcript abundance calculations were automated and performed using the Applied Biosystems 7500 Fast Real-Time PCR System software.

Table 2.3. Primers used for q-PCR reactions.

Name	Primer sequence (5' to 3')
CCA1 – Forward	5'-TCTGTGTCTGACGAGGGTCG-3'
CCA1 – Reverse	5'-ACTTTGCGGCAATACCTCTCTGG-3'
LHY – Forward	5'-CCTTCCGAACATTTCTTTGGT-3'
LHY – Reverse	5'-CAGAGACAAGAGACAAGACATGG-3'
UBQ – Forward	5'-GGCCTTGTATAATCCCTGATGAATAAG-3'
UBQ - Reverse	5'-AAAGAGATAACAGGAACGGAAACATAGT-3'

2.5. Plant growth performance

Plant performance at 17°C and 27°C was evaluated by determining fresh and dry weight of *Arabidopsis* seedlings. Seeds of all accessions were sown onto soil, stratified at 4°C and then grown in a growth room at 22°C under 12:12 L:D conditions. After 12 days seedlings were transplanted into 20-cell half trays with 1 seedling per cell (Figure 2.5). All trays were placed back to the growth room to recover for additional 2 days. Trays were then transferred to a 17°C or 27°C Sanyo MLR350 growth chambers (Sanyo, Osaka, Japan), with 1 tray representing each accession at each temperature. After 14 days all seedlings were harvested and their fresh weight was determined. To obtain dry weight, same seedlings were individually wrapped in foil and placed to the 80°C oven for 48 h.

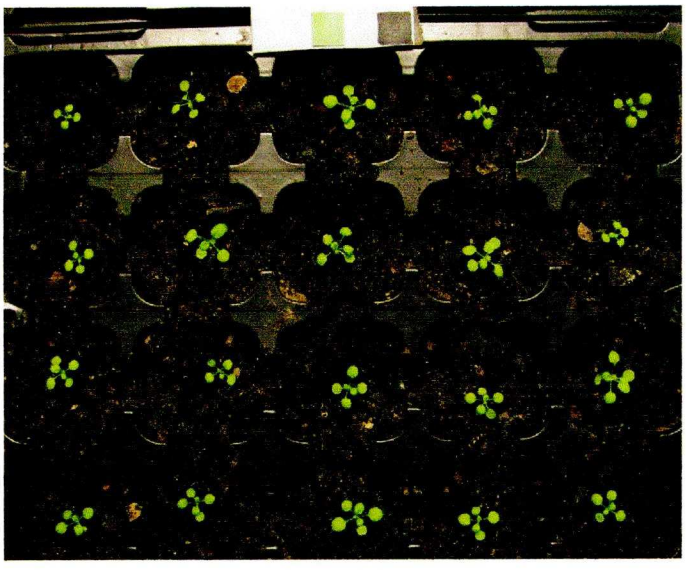


Figure 2.5. Photograph of seedling arrangement for growth performance.

12 day old *Arabidopsis* seedlings are transplanted to 20-well trays. One tray represents each accession at 17°C or 27°C. Green and black coloured boxes correspond to 1 cm^2.

Chapter 3 – Investigation of differentiation between CCA1 and LHY

3.1. Differentiation of the function of the *CCA1* and *LHY* promoters

3.1.1. Introduction:

CCA1 and LHY are MYB-like transcription factors functioning at the core of the *Arabidopsis* circadian clock (Alabadi et al., 2001; Mizoguchi et al., 2002). Both proteins have an almost identical MYB domain, and overall exhibit 42% similarity throughout their amino acid sequences (Figure 1.5 in the introduction section). Overexpresssion of *CCA1* results in the same circadian phenotype as overexpression of *LHY* i.e. hypocotyl elongation, late flowering and abolished rhythmicity in leaf movement, delayed fluorescence and expression of circadian regulated genes (*CAB*, *CCR2*, etc.) (Schaffer et al., 1998; Wang and Tobin, 1998; personal observation). Furthermore, endogenous *CCA1* and *LHY* mRNA and protein abundances cycle in a circadian manner, however, this rhythmicity is abolished in *CCA1*-OX and *LHY*-OX. This suggests that CCA1 and LHY regulate their own and each other's expression. Intriguingly, expression of endogenous *CCA1* and *LHY* in *CCA1*-OX is completely repressed, indicating that CCA1 represses both *LHY* and its own expression. On the other hand, in *LHY*-OX endogenous *LHY* is not completely suppressed and its expression persists at the intermediate levels, indicating that different regulation of *CCA1* and *LHY* is highly likely (Schaffer *et al.*, 1998; Wang and Tobin, 1998). Comparison of the *CCA1* and *LHY* promoters revealed a low level of similarity between the sequences, except for 3 regions of unknown function and a few transcription factor binding sites (Suppl. Figure 1) (Spensley et al., 2009). The *LHY* promoter lacks a CHE transcription factor binding site, which is present in the *CCA1* promoter. CHE has been shown to directly regulate CCA1 by repressing its expression, however, it does not affect LHY. On the other hand, both CCA1 and

LHY can inhibit CHE by binding to the *CHE* promoter (Pruneda-Paz et al., 2009). The difference between *CCA1* and *LHY* regulation could in part contribute to the temperature compensation mechanism of the *Arabidopsis* circadian clock. It has been shown that CCA1 and LHY are involved in buffering the clock against low and high temperatures, respectively (Gould et al., 2006). A loss-of-function *lhy* mutant has a significantly shorter circadian period than the *cca1*-null mutant when subjected to 27°C, underlining the importance of LHY at high temperatures. On the other hand, *cca1*-null has a greater reduction in period length at 12°C in comparison to the *lhy*-null mutant, suggesting an increased necessity for CCA1 under these conditions. Interestingly, the abundance of *LHY* transcript at 27°C is greatly decreased, whilst *CCA1* mRNA levels are unaffected by the temperature change (Gould et al., 2006). The low level of *LHY* expression of at high temperatures might depend specifically upon the functioning of its promoter. However, involvement of other factors, such as RNA degradation rate, could also be possible.

To gain an insight into whether there is a difference between the promoter-mediated regulation of *CCA1* and *LHY*, and if this regulation is temperature dependent, the promoters were swapped between the two genes. Constructs *CCA1::CCA1*and *LHY::LHY* were also created as controls. New transgenes, *CCA1::CCA1* and *LHY::CCA1,* were independently introduced into *cca1*-null (*cca1-11*), while *LHY::LHY* and *CCA1::LHY* were introduced into an *lhy*-null (*lhy-21*) mutant.

First, it was checked whether *CCA1::CCA1* and *LHY::LHY* were able to rescue *cca1*-null and *lhy*-null circadian phenotypes, respectively, at a standard temperature (17°C). It was then checked whether acquired phenotypes were temperature compensated by subjecting the plants to increased temperature (27°C).

Secondly, the circadian phenotype of *cca1* null plants carrying *LHY::CCA1* and *lhy* null with *CCA1::LHY* were examined at 17°C to check whether these mutants could be rescued by *CCA1* and *LHY* expressed from different promoters. To investigate whether regulation from either promoter is temperature dependent, both types of plants were then examined at 27°C.

3.1.2. Results:

3.1.2.1. Building constructs

"Promoter-swapped" constructs were built employing the fusion PCR technique described in Materials and Methods and Szewczyk et al. (2007). First, the *CCA1* promoter, *CCA1* coding region, *LHY* promoter and *LHY* coding region were separately amplified from *Arabidopsis* genomic DNA using specific primers with flanking tails. Due to the current lack of information about the extent of 5'UTRs in the CCA1 and LHY functioning, it was decided to include both, promoters and 5'UTRs, in the current study. Thus, the *CCA1* promoter region consisted of 920 bp of sequence upstream from the ATG translational site, and included the promoter and 5'UTR. The *LHY* promoter region was 1723 bp long and also included the promoter and 5'UTR. Coding regions of *CCA1* and *LHY* encompassed the 3'UTR regions.

PCR products obtained during the first round were purified and fused together in the second round of PCR using nested primers to create the following constructs: *CCA1::CCA1, LHY::LHY, CCA1::LHY* and *LHY::CCA1*. These constructs were subsequently introduced into appropriate mutant plants, i.e. *CCA1::CCA1* and *LHY::CCA1* were introduced into *cca1* null while *LHY::LHY* and *CCA1::LHY* into *lhy* null (Table 3.1). Based on Mendel's segregation rule, transgenic lines that had single insertion loci were identified. Third generation (T_3) seeds of 5 to 6 independent plant lines, per chimeric gene, per mutant plant (*cca1-11* or *lhy-21*), were used for all experiments.

3.1.2.2. Circadian rhythmicity in cca-11 and lhy-21 loss-of-function mutants at 17°C

The first *cca1* loss-of-function mutant (*cca1-1*) was created in the Wassilewskija (Ws) background by a 34-kb T-DNA insertion into the 4th intron of

Table 3.1. Combination of constructs and mutant backgrounds used to create new transgenic plants, as indicated by the "+" sign.

Construct	Plant background:	
	***cca1* null**	***lhy* null**
CCA1::CCA1	+	-
LHY::LHY	-	+
LHY::CCA1	+	-
CCA1::LHY	-	+

the *CCA1* gene (Green and Tobin, 1999). This resulted in no detectable *CCA1* mRNA or protein, suggesting that not even a truncated form of CCA1 was produced. The original *lhy* loss-of-function line (*lhy-11*) was isolated from the *LHY*-OX population mutagenized by EMS (Mizoguchi et al., 2002). The *Lansberg erecta* accession was the background for this *lhy* null mutant. In this study *cca1-11* and *lhy-21* single loss-of-function mutants were used. Both mutants were created by T-DNA insertion into the Ws background plants and were described in Hall et al. (2003). Both were shown not to have functional CCA1 or LHY. A loss of function of either CCA1 or LHY affects circadian outputs such as leaf movement, delayed fluorescence, hypocotyl length, expression of circadian controlled genes and flowering (Green and Tobin, 1999; Alabadi et al., 2002; Mizoguchi et al., 2002; Gould et al., 2009). In this study, the circadian period was assessed by leaf movement assay and supplemented by delayed fluorescence assay. Plants were grown under 12 h light:12 h dark (12:12 L:D) cycles for 10 days for leaf movement or 15 days for delayed fluorescence and then transferred to constant light where their free-running circadian rhythms were measured. The luciferase reporter gene was not used as it would require transformation of multiple transgene lines and would exceed the available time frame.

Plants with loss-of-function in CCA1 or LHY resulted in shorter period when compared to the wild type (leaf movement: Ws – 26.22, *cca1-11* – 24.23, *lhy-21* – 24.73; delayed fluorescence: Ws – 25.22, *cca1-11* – 23.38, *lhy-21* – 23.66) (Figure 3.1). However, the robustness of the clock was not affected by the loss of either. This is indicated by plotting period estimates for individual data points against their relative amplitude errors (Rel. Amp. Error or RAE) (Figure 3.1 A, B and D).

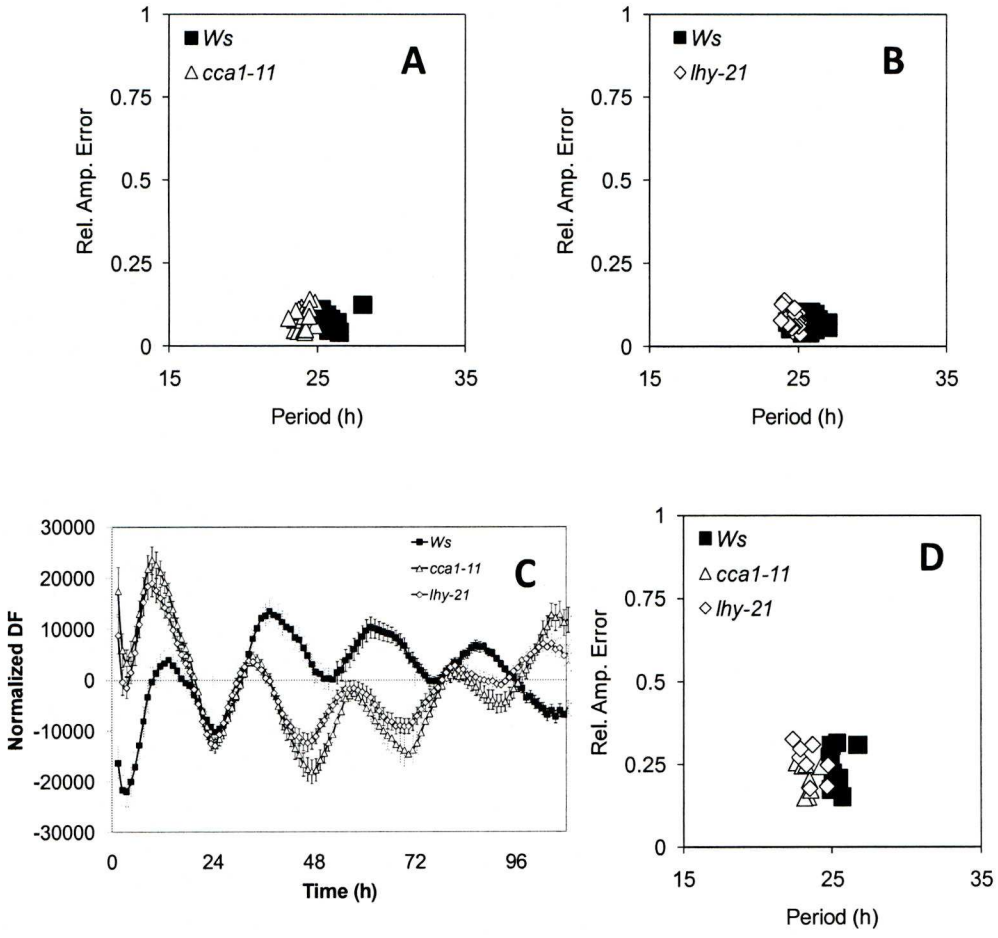


Figure 3.1. Analysis of circadian phenotypes for *cca1-11* and *lhy-21*

(A) and (B) – Leaf movement. Period estimates for individual leaves plotted against relative amplitude error (Rel. Amp. Error) for *cca1-11* (A) and *lhy-21* (B) against Ws. Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C and continuous light, at which leaf movement rhythms were assessed, n=30.

(C) and (D) – Delayed fluorescence. (C) Normalized averages +/- SE for delayed fluorescence of wild-types Ws, *cca1-11* and *lhy-21*. (D) Circadian period estimates for delayed fluorescence plotted against Rel. Amp. Error. Delayed fluorescence was monitored under continuous light at 22°C from 15 days old seedlings. Average was calculated from n=8. All experiments were independently repeated at least twice with similar results.

RAE is a measure of rhythm robustness which varies from 0 (robust rhythm) to 1 (no rhythm) (Dowson-Day and Millar, 1999). Taken together, CCA1 or LHY loss-of-function causes circadian period shortening, which is consistent with previous reports (Mizoguchi et al., 2002; Gould et al., 2009).

3.1.2.3. Introduction of CCA1::CCA1 and LHY::LHY to cca1 and lhy nulls

To check if chimeric genes, generated by fusion PCR, were functional, *CCA1::CCA1* and *LHY::LHY* constructs were created and introduced into *cca1-11* and *lhy-21* mutants, respectively. Since *cca1-11* and *lhy-21* display short period circadian rhythms (Figure 3.1), it was expected that introduction of *CCA1::CCA1* and *LHY::LHY* into these mutants would rescue the short period phenotypes. To test this hypothesis, 6 single loci independent transgenic lines of the *CCA1::CCA1*-in-*cca1* null background, and 6 lines of the *LHY::LHY*-in-*lhy* null were subjected to leaf movement assay. Wild-type (Ws), *cca1* and *lhy* nulls were used as controls. All plants were grown under 12:12 L:D cycles for 10 days and then transferred to constant light where their free-running periods were measured. Leaf movement data was plotted as RAE graphs, which shows both, the variability of period estimates as well as the robustness of the rhythm. A wide spread of data points on the graph indicates a loss in the precision of the clock, while tightly clustered data points with low RAE associate with robust rhythms and a precise clock (Gould *et al.*, 2006). To determine whether circadian outputs in examined lines were governed by abundance of *CCA1* and *LHY*, the quantity of *CCA1* and *LHY* transcripts were measured. Based on the reasoning that maximum expression of these two genes occurs in the morning (Lu *et al.*, 2009), tissue was harvested 1 h after dawn from plants entrained to 12:12 L:D. Transcript levels were measured by quantitative real-time PCR (see Materials and Methods). Abundance of *CCA1* and *LHY* transcripts from *CCA1::CCA1*-in-*cca1*

null and *LHY::LHY*-in-*lhy* null transgenic lines was then plotted with the leaf movement data for comparison. Furthermore, all transformed lines were subjected to the delayed fluorescence assay. Delayed fluorescence data mirrored leaf movement results and is presented in the supplementary data (Suppl. Figure 2).

Out of 6 lines, used to assess the effect of *CCA1::CCA1* on *cca1* null and subjected to leaf movement assay, lines 2, 4 and 9 had a period matching the wild type (Table 3.2). This data was supported by *CCA1* expression measured in these lines, which was only slightly higher than the wild-type control (Figure 3.2 B, C and F). In contrast, line 1 failed to rescue the loss-of-function mutant, possibly due to very low levels of *CCA1* transcripts (Figure 3.2 A). Two transgenic lines, 7 and 8, were also assigned a short period. However, in this case, almost half of the seedlings were scored by BRASS software as arrhythmic, whilst another half had an increased relative amplitude error. In addition, the standard deviation of the periods was relatively high, indicating inconsistency in circadian period between the seedlings. Therefore, lines 7 and 8 were believed to be arrhythmic. Transcript levels of *CCA1* and *LHY* in these lines were slightly lower than in the wild-type (Figure 3.2 D and E).

In comparison, *LHY::LHY* completely rescued lines 4 and 6 of *lhy* null. *LHY* abundance in these lines was either equal (line 4) or only slightly lower (line 6) than the control (Figure 3.3 C and D). Lines 2, 7 and 8 exhibited a 2 h longer period than the wild type (Table 3.2), which correlated with increased levels of *LHY* (Figure 3.3 A, E and F). Line 3 was arrhythmic, and had low amounts of *LHY*. Interestingly, all *LHY::LHY*-in-*lhy* null lines had decreased levels of *CCA1*, indicating a possible repression of *CCA1* expression.

Table 3.2. Leaf movement period for transgenic lines carrying *CCA1::CCA1*-in-*cca1* null and *LHY::LHY*-in-*lhy* null estimated at 17°C.

Name	Line Number	Period (h)	SD	n
Ws		26.2	0.5	29
cca1-11		24.2	0.3	30
lhy-21		24.7	0.3	28
CCA1::CCA1				
	L.1	23.8	0.3	30
	L.2	26.3	0.4	29
	L.4	26.5	0.6	29
	L.7	22.4	2.7	21
	L.8	22.8	0.8	16
	L.9	27.0	0.5	29
LHY::LHY				
	L.2	28.9	0.9	25
	L.3	19.4	3.5	24
	L.4	26.8	1.0	27
	L.6	26.3	0.5	29
	L.7	28.5	0.6	30
	L.8	28.1	0.7	30

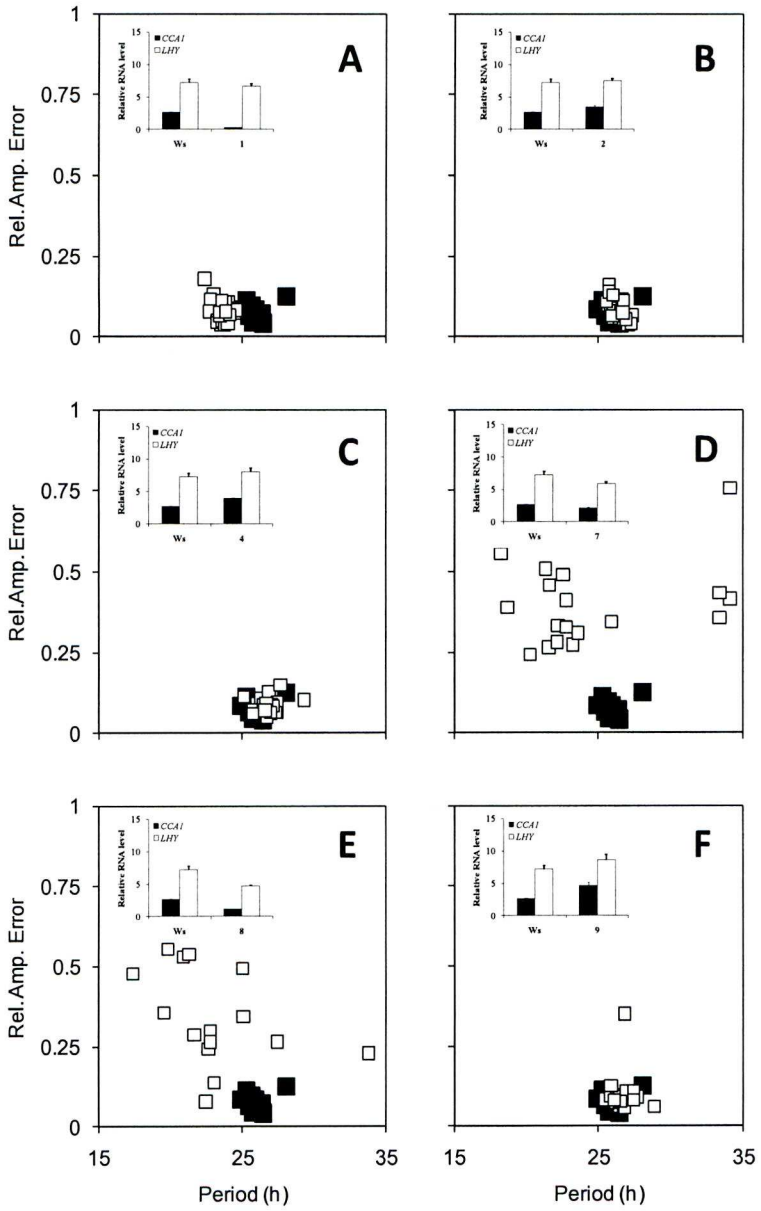


Figure 3.2. Analysis of the leaf movement and gene expression for *CCA1::CCA1*-in-*cca1* null transgenic plants. *CCA1::CCA1* chimeric gene either fails to rescue (A), completely rescues (B, C and F) or causes arrhythmia (D and F) in *cca1* null plants.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its relative amplitude error (Rel. Amp. Error). Black squares, wild type Ws (n=30), open squares, transgenic line (n=30). Individual transgenic lines 1, 2, 4, 7, 8 and 9 are plotted on graphs A to F respectively. Inset bar charts within each graph illustrate transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene). All plants were grown on MS agar with 3% sucrose under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and subjected to qRT-PCR analysis.

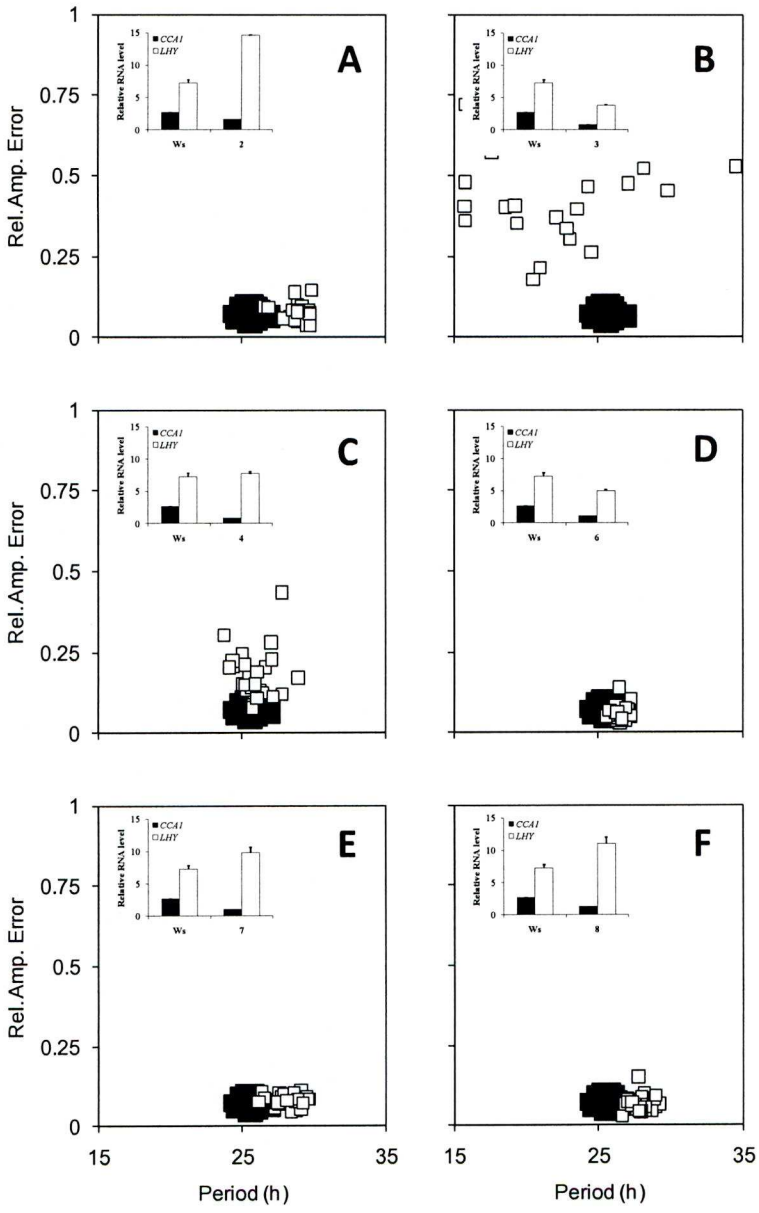


Figure 3.3. Analysis of the leaf movement and gene expression of *LHY::LHY*-in-*lhy* null transgenic plants. *LHY::LHY* chimeric gene either rescues (C and D) or causes long period phenotype (A, E and F) or arrhythmia (B) in *lhy* null plants.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against Rel. Amp. Error. Black squares, wild type Ws (n=30), open squares, transgenic line (n=30). Individual transgenic lines 2, 3, 4, 6, 7 and 8 are plotted on graphs A to F respectively. Inset bar charts within each graph illustrate transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene). All plants were grown on MS agar with 3% sucrose under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and subjected to qRT-PCR analysis.

To summarize, the arrhythmic phenotypes of *CCA1::CCA1*-in-*cca1* null lines 7 and 8 and *LHY::LHY*-in-*lhy* null line 3 could be attributed to overproduction of CCA1 and LHY caused by introduction of several tandem copies of the transgene into the same genome, a common event in transformation of plants (De Buck et al., 2004). These lines also exhibited elongated hypocotyls and petiols (data not shown), which are also characteristic of *CCA1* and *LHY* overexpression in *Arabidopsis* (Schaffer et al., 1998; Wang and Tobin, 1998).

Increased *LHY* abundance in *LHY::LHY*-in-*lhy* null lines coincided with the period lengthening of circadian outputs and also with decreased levels of *CCA1*. In the case of *CCA1::CCA1*-in-*cca1* null, lines that expressed *CCA1* and *LHY* at amounts slightly higher than the wild-type, exhibited a circadian period similar to the control (Figure 3.2 B, C, F). However, a decrease in *CCA1* and *LHY* levels in *LHY::LHY* -in-*lhy* null lines also resulted in the wild-type period (Figure 3.3 C and D). This suggests that circadian clock is buffered against changes in *CCA1* and *LHY* mRNA. Since *CCA1* and *LHY* are functionally redundant, it is possible that a combined rather than individual amount of *CCA1* and *LHY* transcripts is of more significance.

3.1.2.4. Introduction of LHY::CCA1 to cca1 null and CCA1::LHY to lhy null

To examine whether the *CCA1* and *LHY* promoters are functionally different, *LHY::CCA1* and *CCA1::LHY* constructs were created. This was achieved by fusing a 920 bp *CCA1* promoter region to the genomic sequence of the LHY protein coding region at the ATG translation start site. Similarly, 1723 bp *LHY* promoter region was joined to the coding region of *CCA1*. The *LHY::CCA1* and *CCA1::LHY* were then separately introduced into *cca1* null and *lhy* null, respectively, to create *LHY::CCA1*-in-*cca1* null and *CCA1::LHY*-in-*lhy* null transgenic plants (Table 3.1).

When leaf movement, delayed fluorescence and *CCA1/LHY* expression levels of *LHY::CCA1*-in-*cca1* null plants were measured, it was observed that these transgenic lines almost completely matched the wild type control in all measured outputs (Table 3.3; Figure 3.4; Suppl. Figure 3 A). Even though some variation in transcript levels (mostly in *CCA1*) was observed amongst the lines (e.g. Figure 3.4 D, E and F), it only slightly, if at all, affected the circadian period in these plants.

On the other hand, *CCA1::LHY*-in-*lhy* null plants had a phenotype entirely different than the wild type (Figure 3.5). Line 6 had a short circadian period (Figure 3.5 B) and analysis of gene expression detected no *LHY* mRNA. This phenotype matched the phenotype of the *lhy* null background, suggesting unsuccessful transformation event of this line with the *CCA1::LHY*. It is possible that transformation of this line was aberrant, and while transformed plants received the antibiotic resistance cassette, the actual gene of interest appeared to be absent or not expressed. Interestingly, the remaining 4 lines from the *CCA1::LHY*-in-*lhy* null transformation also exhibited a short circadian period, however, in all cases the period was more than 5 h shorter than of the *lhy* null mutant (Table 3.3; Figure 3.5 and Suppl. Figure 3 B). Furthermore, analysis of the *CCA1* and *LHY* expression in these transformants revealed an almost complete reduction of the *CCA1* and *LHY* transcripts, suggesting transcriptional repression of both of these genes (Figure 3.5 A, C, D and E).

To summarize, comparison of *LHY::CCA1*-in-*cca1* null and *CCA1::LHY*-in-*lhy* null plants suggests that *CCA1* and *LHY* promoter activity is different. The *LHY* promoter in *LHY::CCA1*-in-*cca1* null can produce *CCA1* mRNA at amounts similar to that found in the wild type. Hence, *LHY::CCA1* can complement *cca1* null plants.

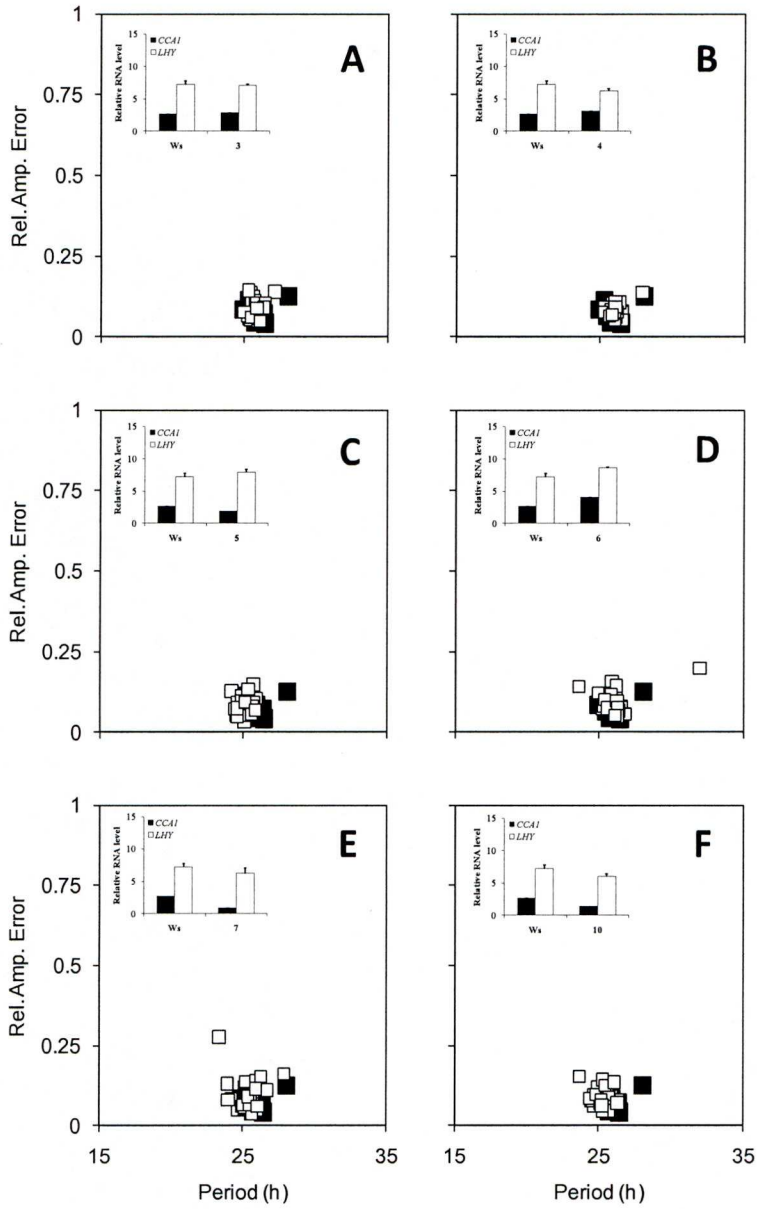


Figure 3.4. Analysis of the leaf movement and gene expression of *LHY::CCA1*-in-*cca1* null transgenic plants. *LHY::CCA1* almost completely rescues *cca1* null plants.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, wild type Ws (n=30), open squares, transgenic line (n=30). Individual transgenic lines 3, 4, 6, 7 and 10 are plotted on graphs A to F respectively. Inset bar charts within each graph illustrate transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene). All plants were grown on MS agar with 3% sucrose under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and subjected to qRT-PCR analysis.

Table 3.3. Leaf movement period for transgenic lines carrying *LHY::CCA1* in *cca1* null and *CCA1::LHY* in *lhy* null estimated at 17°C.

Name	Line Number	Period (h)	SD	n
Ws		26.2	0.5	29
cca1-11		24.2	0.3	30
lhy-21		24.7	0.3	28
LHY::CCA1				
	L.3	25.6	0.3	29
	L.4	25.6	0.4	29
	L.5	25.3	0.4	29
	L.6	25.7	0.7	30
	L.7	25.4	0.4	27
	L.10	25.2	0.4	29
CCA1::LHY				
	L.3	18.6	1.4	29
	L.6	24.5	0.3	30
	L.9	19.0	0.8	29
	L.11	18.3	0.8	30
	L.14	19.7	1.0	29

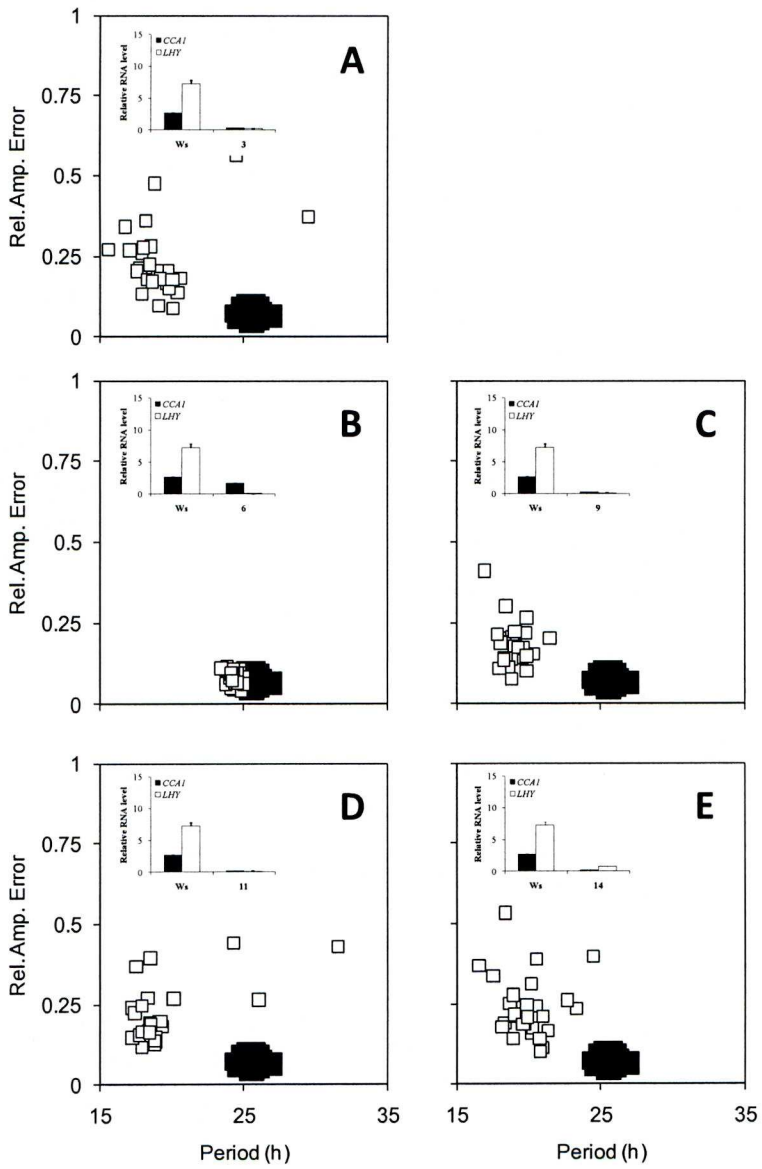


Figure 3.5. Analysis of the leaf movement and gene expression of *CCA1::LHY*-in-*lhy* null transgenic plants. *CCA1::LHY* causes a short period phenotype in *lhy* null plants.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, wild type Ws (n=30), open squares, transgenic line (n=30). Individual transgenic lines 3, 6, 9, 11 and 16 are plotted on graphs A to E respectively. Inset bar charts within each graph illustrate transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene). All plants were grown on MS agar with 3% sucrose under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and subjected to qRT-PCR analysis.

On the other hand, *CCA1::LHY*-in-*lhy* null transgenic plants, where *LHY* is expressed from the *CCA1* promoter, experience suppression of *LHY* and endogenous *CCA1*, producing a very short period phenotype. This phenotype matches a complete *cca1-11 lhy-21* double mutant described in Lu *et al.* (2009), which displayed a rhythm of approximately 19 h. As CCA1 and LHY are known to inhibit their own and each other's transcription (Green and Tobin, 1999), a double null mutant phenotype could be explained by an over-production of *LHY* driven by the *CCA1* promoter, which would subsequently lead to the suppression of any expression of *CCA1* and *LHY*. This, in turn, would result in a *cca1:lhy* double null mutant phenotype.

It is highly probable that a combination of factors, such as promoter activity and mRNA stability, is involved in determining circadian phenotype. *CCA1* transcripts are known to be unstable (Yakir et al., 2007a). Therefore, *LHY* mRNA is more stable than *CCA1* mRNA, and if the *LHY* promoter is less active than the *CCA1*, abundance of the *CCA1* acquired from the *LHY* promoter would be less than in the wild-type. However, experimental data of the current study shows that such *CCA1* expression is sufficient to maintain normal circadian rhythmicity (Figure 3.4). On the other hand, *CCA1*::*LHY* would result in rapidly increased amounts of stable *LHY* transcript. Kim et al. (2003) showed that expression of endogenous *CCA1* is more suppressed than expression of endogenous *LHY* in *LHY*-OX, suggesting stronger repression of *CCA1* by *LHY*. Hence, increased amounts of stable *LHY* transcript in *CCA1::LHY*-in-*lhy* null plants could cause a prompt inhibition of genes expressed from the *CCA1* promoter and unbalance the clock. Further work is required to address this hypothesis.

3.1.2.5. Circadian rhythmicity in cca-11 and lhy-21 loss-of-function mutants at 27°C

Gould et al. (2006) have proposed that CCA1 and LHY play different roles in temperature compensation. This was suggested after circadian periods of *CAB::LUC* in *cca1* and *lhy* null mutants were analyzed under different temperature conditions. Circadian rhythms of both mutants oscillated with the same period at normal temperature (17°C). However, the period difference between the *cca1* and *lhy* nulls occurred when plants were subjected to high (27°C) or low temperature (12°C).

To confirm the Gould *et al.* (2006) observations, *cca1-11* and *lhy-21* circadian phenotypes were analyzed at a high temperature of 27°C using leaf movement assay. Ws was used as a control. Plants were first grown at 22°C and 12:12 L:D cycles, and after 10 days transferred to constant light at 17°C or 27°C, where their free-running period was measured. In addition, accumulation of *CCA1* and *LHY* transcripts at 17°C and 27°C was also examined. For this, plants were grown at 22°C 12:12 L:D for 7 days and then transferred to either 17°C or 27°C for 3 additional days. Plant tissue was harvested 1 h after the lights turned on.

No big difference between 17°C and 27°C was observed when leaf movement rhythmicity was assessed in the Ws control (17°C – 26.22; 27°C – 25.62), indicating that its circadian period was well temperature compensated (Figure 3.6). Interestingly, high temperature had an inhibiting effect on *CCA1* and *LHY* mRNA levels, decreasing them by almost a half (Figure 3.6 B). Contrary to the control, *cca1-11* and *lhy-21* circadian rhythms were not as well temperature compensated. Both *cca1* and *lhy* are short period mutants, however, with the temperature increase their circadian periods decreased even further (Figure 3.6 C and E). A decrease in *CCA1* and *LHY* transcript levels was also observed, with *CCA1* responding to the high temperature more strongly than *LHY* (Figure 3.6 D and F).

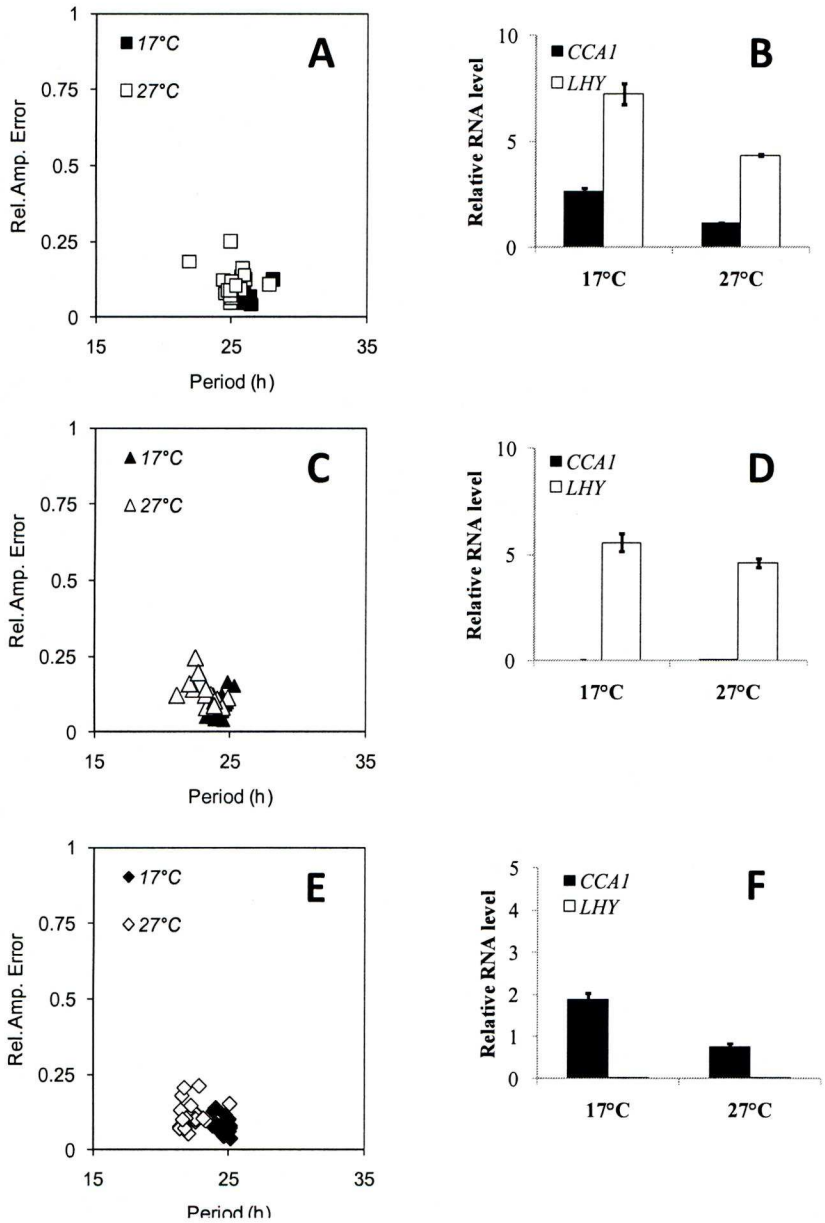


Figure 3.6. Effect of 27°C on leaf movement period and *CCA1* and *LHY* expression levels.

Plants were grown on MS agar supplemented with 3% sucrose at 22°C under 12:12 L:D for 10 days before the transfer to continuous light at 17°C or 27°C, where leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its relative amplitude error (Rel. Amp. Error) for A - wild type Ws (n=20), C – *cca1-11* (n=20) and E – *lhy-21* (n=20). Transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene) at 17°C and 27°C was measured in Ws (B), *cca1-11* (D) and *lhy-21* (F). All plants were grown on MS agar with 3% sucrose at 22°C under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C or 27°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and was subjected to qRT-PCR analysis.

Despite this result, it was *lhy*- null that had a more profound response to the temperature change and displayed a ~1 h shorter period than the *cca1*-null (*lhy* – 22.18; *cca1* – 23.65). Taken together, leaf movement was well temperature compensated in the Ws, while circadian phenotypes of *cca1* and *lhy* mutants were temperature dependant. This conclusion is consistent with Gould et al. (2006). However, contrary to the Gould et al. (2006) data on temperature independent *CCA1* expression, it was found that high temperature had a negative effect and decreased transcript levels of both *CCA1* and *LHY*. Such a variation in results could be explained by differences in experimental design. In this study plant tissue was harvested at dawn after 12:12 L:D entrainment, while Gould et al. (2006) measured the expression profile of *CCA1* and *LHY* over the course of 24 h after plants free-ran for 3 days under continuous light.

To summarize, decreased amounts of *CCA1* and *LHY* in response to high temperature do not affect circadian leaf movement in Ws, indicating that either the remaining amount of transcripts is enough to keep circadian leaf movement buffered against the temperature change, or other genes/proteins are involved in the temperature compensation mechanism. It is also possible that both of these systems work together.

3.1.2.6. Effect of 27°C on CCA1::CCA1-in cca1-null and LHY::LHY-in-lhy null plants

Circadian phenotypes of *CCA1* and *LHY* loss-of-function mutants appeared to be temperature dependant (Figure 3.6). To check whether circadian rhythmicity of the *cca1* and *lhy* null mutants, carrying *CCA1::CCA1* and *LHY::LHY*, respectively, is also temperature dependant or if it is as well temperature compensated as the wild type control is, circadian phenotype of the transformants were assessed at 17°C and

27°C using leaf movement. Levels of *CCA1* and *LHY* expression were also measured by harvesting plant tissue from seedlings grown under 12:12 L:D light conditions at 22 °C and after 1 week transferred to 17°C or 27°C. Plant tissue was harvested on the 4th day after the transfer, 1 h after the lights turned on. Delayed fluorescence was not used for the circadian assessment at 27°C as DF rhythms were less robust in the Ws control making data from transgenic lines difficult to interpret.

First, temperature compensation was tested in *CCA1::CCA1*-in-*cca1* null transplants. All *CCA1::CCA1*-in-*cca1* null lines were able to maintain the same leaf movement circadian phenotype at 27°C as they did at 17°C (Table 3.2, 3.4 and Figure 3.7). Lines 2, 4 and 9 sustained robust rhythms even though levels of *CCA1* and *LHY* mRNA decreased with the temperature increase (Figure 3.7). Lines 7 and 8 remained arrhythmic. Interestingly, high temperature completely changed the expression profile of *CCA1* and *LHY* in these two lines (Figure 3.7). Commonly, levels of *LHY* are higher than that of *CCA1* (Lu et al., 2009). Here, on the other hand, subjection of line 7 and 8 plants to high temperature resulted in an increase in *CCA1* levels and a decrease in *LHY*.

In comparison to *CCA1::CCA1*-in-*cca1* null mutants, high temperature caused a change in circadian phenotype in *LHY::LHY*-in-*lhy* null transplants. The variance in circadian period in almost all transgenic lines increased with the temperature increase, which is indicated by the large spread of data points along the X-axis (Figure 3.8). Furthermore, line 2, which exhibited robust circadian rhythms at 17°C, became arrhythmic at 27°C. As for *CCA1* and *LHY* expression data, high temperature did not have a major affect on mRNA levels. Only line 3 showed an increase in *LHY* mRNA levels, while in the rest of the lines levels of *CCA1* and *LHY* remained the same or slightly decreased (Figure 3.8).

Table 3.4. Leaf movement period estimates for 27°C for *CCA1::CCA1*-in-*cca1* null and *LHY::LHY* -in-*lhy* null transgenic lines

Name	Line Number	Period (h)	SD	n
Ws		24.9	0.7	16
cca1-11		23.7	1.1	14
lhy-21		22.2	0.5	19
CCA1::CCA1				
	L.1	22.8	0.8	19
	L.2	25.4	0.9	14
	L.4	25.2	1.8	15
	L.7	20.9	4.7	14
	L.8	24.9	5.4	18
	L.9	26.6	1.0	15
LHY::LHY				
	L.2	21.8	6.8	16
	L.3	24.1	4.0	11
	L.4	26.2	1.7	11
	L.6	25.6	1.8	14
	L.7	26.6	1.4	11
	L.8	26.1	1.7	18

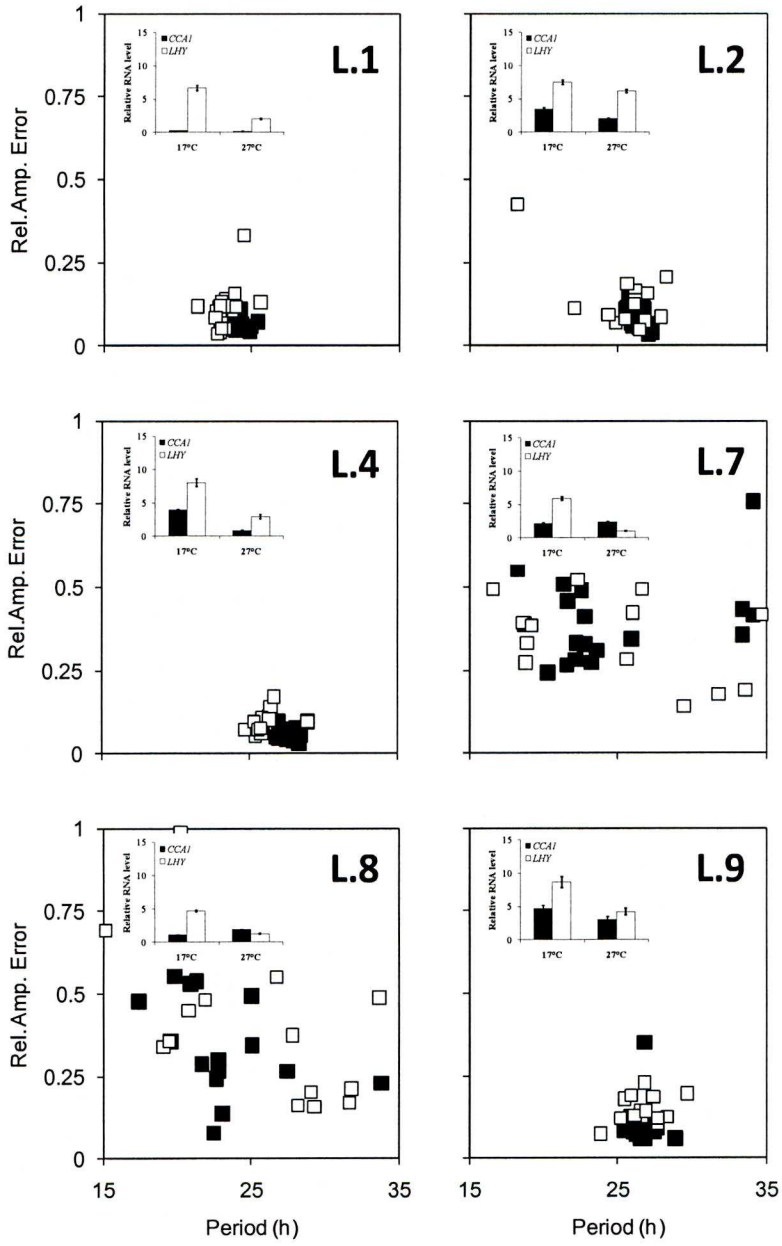


Figure 3.7. Effect of 27°C on circadian phenotype of *CCA1::CCA1*-in-*cca1* null transformants. Circadian phenotype was assessed by leaf movement period and *CCA1* and *LHY* expression levels.

Plants were grown on MS agar supplemented with 3% sucrose at 22°C under 12:12 L:D for 10 days before the transfer to continuous light at 17°C or 27°C, where leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error.Black squares, transgenic line at 17°C (n=30), open squares, transgenic line at 27° C (n=30). Transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene) was measured at 17°C and 27°C. All plants were grown on MS agar with 3% sucrose at 22°C under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C or 27°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and was subjected to qRT-PCR analysi

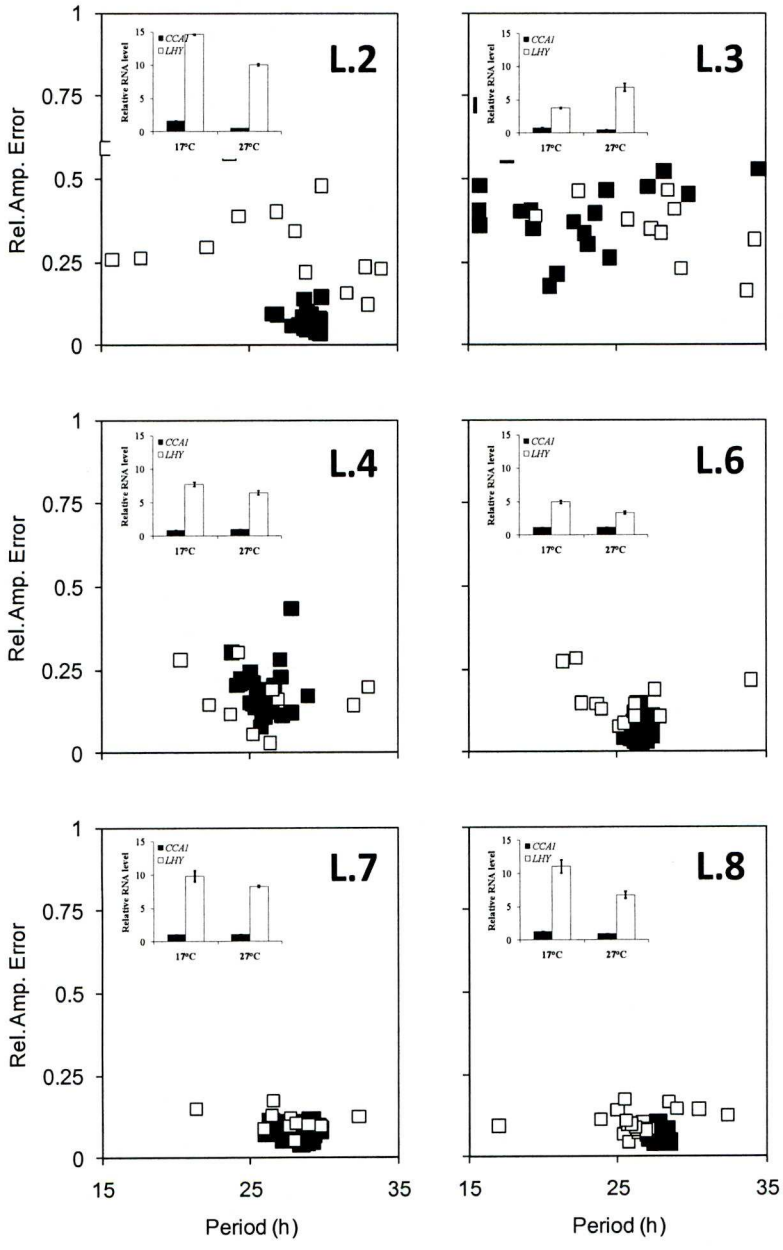


Figure 3.8. Effect of 27°C on circadian phenotype of *LHY::LHY*-in-*lhy* null transformants. Circadian phenotype was assessed by leaf movement period and *CCA1* and *LHY* expression levels.

Plants were grown on MS agar supplemented with 3% sucrose at 22°C under 12:12 L:D for 10 days before the transfer to continuous light at 17°C or 27°C, where leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, transgenic line at 17°C (n=30), open squares, transgenic line at 27° C (n=30). Transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene) was measured at 17°C and 27°C. All plants were grown on MS agar with 3% sucrose at 22°C under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C or 27°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and was subjected to qRT-PCR analysis.

Overall, circadian phenotypes of the *CCA1::CCA1*-in-*cca1* null lines were better temperature compensated than those of *LHY::LHY*-in-*lhy* null plants. In general, temperature increase caused a decrease in *CCA1* and *LHY* abundance. However, *CCA1::CCA1*-in-*cca1* null lines arrhythmic at both 17°C and 27°C had an increase in *CCA1* levels at 27°C. On the other hand, *LHY::LHY*-in-*lhy* null line 3, which was also arrhythmic at both temperatures, also showed an increase in *LHY* levels with the temperature increase. This suggests that arrhythmicity in these lines is most probably caused by improper functioning or regulation of *CCA1/LHY*, with position effect in the genome during the transformation event being one of the possible explanations.

In summary, the obtained results suggest that *LHY::LHY* in-*lhy* null plants are more sensitive to the temperature increase than *CCA1::CCA1*-in-*cca1* null plants. It is not clear whether the observed temperature response could be related to increased sensitivity to the changeable *LHY* or initially low *CCA1* levels.

Surprisingly, no overall connection could be observed between the one time point measurement of *CCA1* and *LHY* expression at 27°C and the circadian phenotype across both *CCA1::CCA1-in-cca1* null and *LHY::LHY-in-lhy* null plants indicating that either 1-time point harvest was not sufficient to give an insight to the correlation between the *CCA1* and *LHY* expression and the circadian phenotype, or expression levels of these genes could not fully determine the phenotype at 27°C.

3.1.2.7. Effect of the 27°C on LHY::CCA1-in-cca1 null and CCA1::LHY-in-lhy null plants

To investigate temperature compensation of the *LHY::CCA1*-in-*cca1* null and *CCA1::LHY*-in-*lhy* null transformants, their circadian leaf movement was examined at 17°C and 27°C. Temperature effect on *CCA1* and *LHY* mRNA levels was also examined as described in 3.1.2.6 section.

At 27°C *LHY::CCA1*-in-*cca1*-null plants were able to maintain robust rhythms of leaf movement. Nevertheless, a raise in temperature did have a mild effect on circadian rhythmicity and slightly shortened the leaf movement circadian period. Even though this period shortening was not noticeable from the BRASS period analysis, it was observed when individual data points were graphed on RAE plots (Table 3.5 and Figure 3.9). As already previously observed in Ws as well as majority of the *CCA1::CCA1*-in-*cca1* null and *LHY::LHY*-in-*lhy* null lines, the levels of *CCA1* and *LHY*, measured at the peak of their expression (1 h after dawn), decreased with the temperature increase. All *LHY::CCA1*-in-*cca1* null lines also exhibited a drop in *CCA1* and *LHY* mRNA, with the latter showing a greater change (Figure 3.9, inset bar charts). While lines 3 and 4 showed a mild decrease in *LHY* abundance, in the rest of the lines amounts of the transcript dropped several folds. However, the changed abundance of *CCA1* and *LHY* did not lead to the loss of the temperature compensation, and the period change observed in *LHY::CCA1*-in-*cca1* null lines is comparable to the change observed in the Ws subjected to the 27°C, suggesting that either amount of the circadian genes transcript in *LHY::CCA1*-in-*cca1* null was sufficient, or, alternatively, other genes/proteins are involved in the temperature compensation mechanism. Again, 1 time-point could be insufficient to capture the influence of transcript abundance change on the circadian phenotype.

Table 3.5. Leaf movement period estimates for 27°C for *LHY::CCA1*-in-*cca1* null and *CCA1::LHY*-in-*lhy* null transgenic lines.

Name	Line Number	Period (h)	SD	n
Ws		24.9	0.7	16
cca1-11		23.7	1.1	14
lhy-21		22.2	0.5	19
LHY::CCA1				
	L.3	24.4	0.9	18
	L.4	23.8	0.6	19
	L.5	24.3	0.7	18
	L.6	24.3	0.8	17
	L.7	24.2	0.8	19
	L.10	24.1	0.9	15
CCA1::LHY				
	L.3	22.8	5.1	14
	L.6	21.6	0.7	18
	L.9	19.9	5.3	12
	L.11	26.6	7.0	17
	L.14	22.3	2.4	11

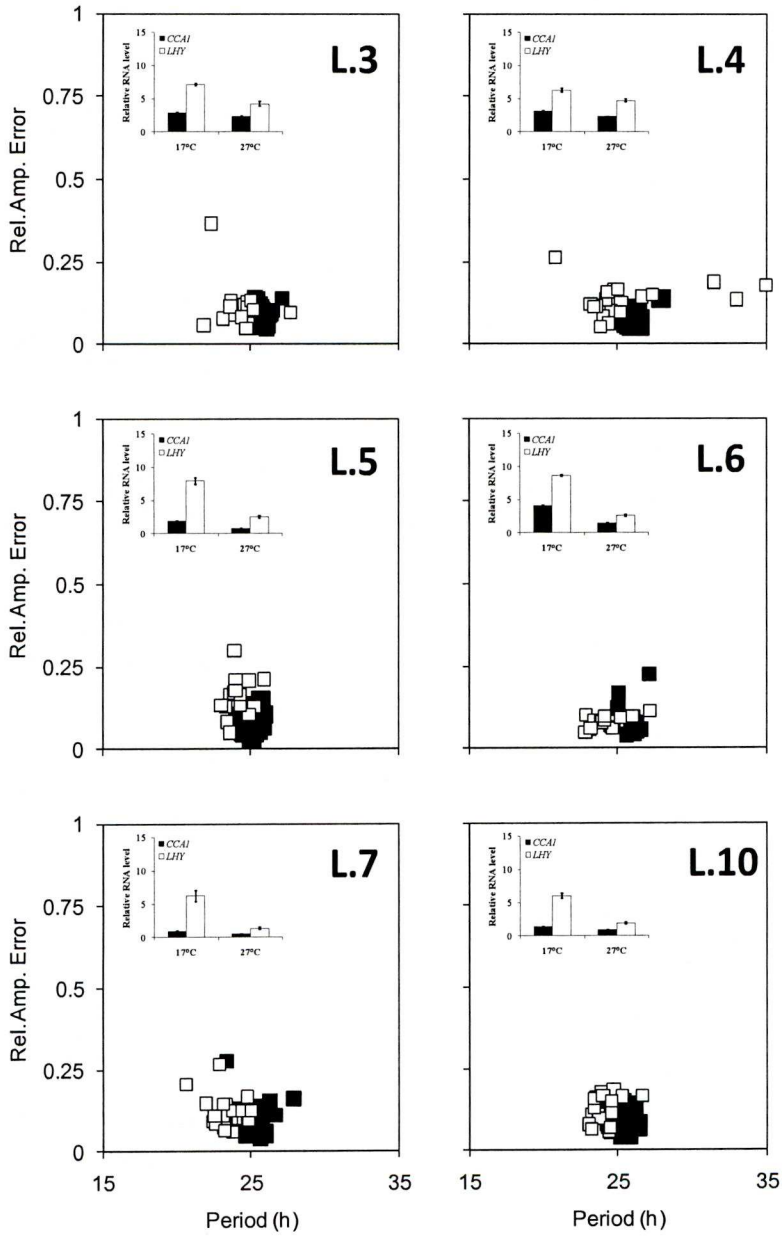


Figure 3.9. Effect of 27°C on circadian phenotype of *LHY::CCA1*-in-*cca1* null transformants. Circadian phenotype was assessed by leaf movement period and *CCA1* and *LHY* expression levels.

Plants were grown on MS agar supplemented with 3% sucrose at 22°C under 12:12 L:D for 10 days before the transfer to continuous light at 17°C or 27°C, where leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, transgenic line at 17°C (n=30), open squares, transgenic line at 27° C (n=30). Transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene) was measured at 17°C and 27°C. All plants were grown on MS agar with 3% sucrose at 22°C under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C or 27°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and was subjected to qRT-PCR analysis.

In comparison to *LHY::CCA1*-in-*cca1* null, *CCA1::LHY*-in-*lhy* null transplants were not able to maintain their circadian rhythmicity at 27°C. Expressing a short period phenotype at 17°C, exposure of these lines to the 27°C caused a considerable increase in the standard deviation of the average circadian period estimate (Table 3.5), indicating that the synchrony in leaf movement between the seedlings and even between leaves of individual seedlings was lost. The loss of *CCA1::LHY*-in-*lhy* null plants' ability to maintain the rhythm could also be observed from the RAE graphs in Figure 3.10. As for the temperature effect on *CCA1* and *LHY* transcript levels in *CCA1::LHY*-in-*lhy* null lines, except line 6, which kept *lhy* null phenotype after the transformation, all other lines showed an increase in *CCA1* expression (Figure 3.10). This observation contradicts the rest of the results, where amounts of *CCA1* and *LHY* decrease with the temperature increase. On the other hand, *CCA1::CCA1*-in-*cca1* null (Figure 3.7) and *LHY:LHY*-in-*lhy* null (Figure 3.8) lines arrhythmic at 17°C and 27°C also exhibited an increase in *CCA1* or *LHY* mRNA with the temperature increase. These results suggest that accumulation of *CCA1* and *LHY* transcript is a temperature dependant process, even though it is unclear why a temperature rise causes a decrease in mRNA abundance in some cases, but an increase in others. It is, however, very likely that a temperature induced increase in *CCA1* or *LHY*, as opposed to a decrease, and arrhythmicity in leaf movement are linked.

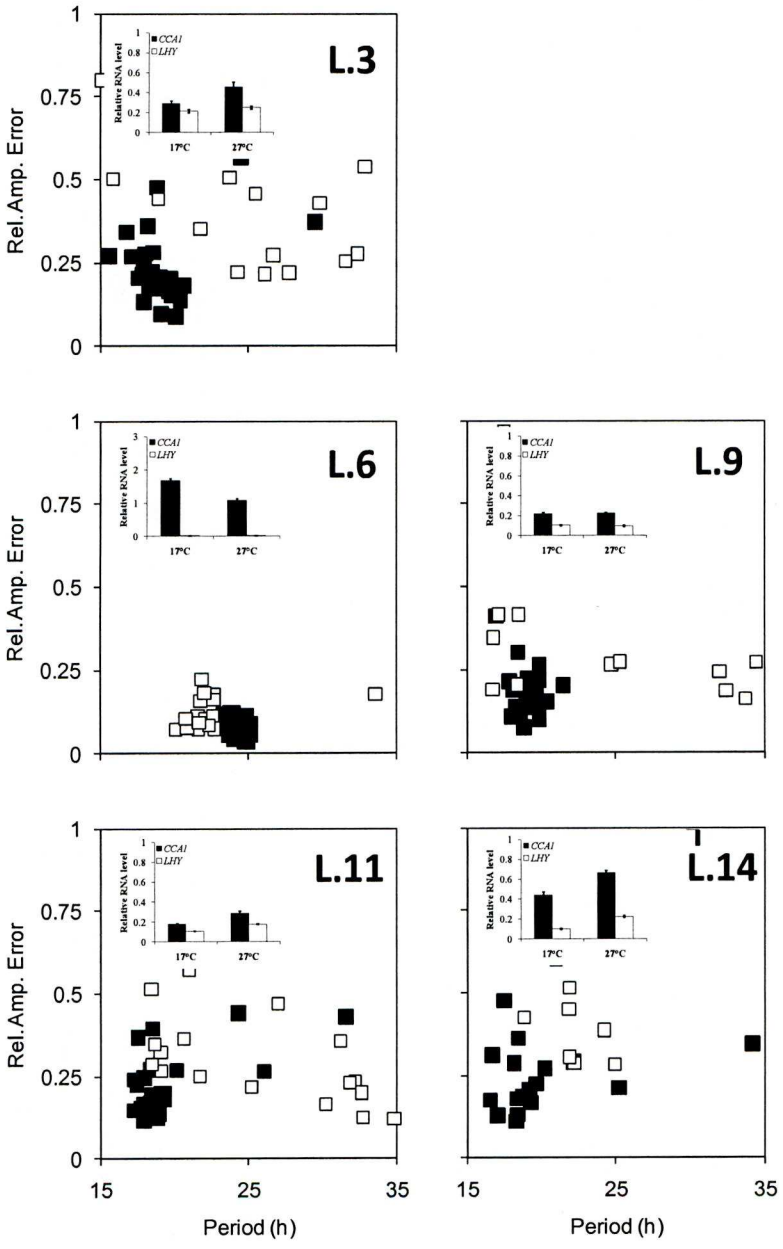


Figure 3.10. Effect of 27°C on circadian phenotype of *CCA1::LHY*-in-*lhy* null transformants. Circadian phenotype was assessed by leaf movement period and *CCA1* and *LHY* expression levels.

Plants were grown on MS agar supplemented with 3% sucrose at 22°C under 12:12 L:D for 10 days before the transfer to continuous light at 17°C or 27°C, where leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, transgenic line at 17°C (n=30), open squares, transgenic line at 27° C (n=30). Transcript abundance for *CCA1* and *LHY* relative to *UBQ10 (*a housekeeping gene) was measured at 17°C and 27°C. All plants were grown on MS agar with 3% sucrose at 22°C under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C or 27°C under 12:12 L:D. Plant tissue was harvested an hour after dawn and was subjected to qRT-PCR analysis.

Furthermore, when Lu et al. (2009) compared a *cca1-1 lhy-11* double knockout mutant expressing a truncated LHY protein with a truly double null *cca1-1 lhy-21*, it was observed that the mutant with no functional CCA1 or LHY had a very short circadian period (19 h), while the mutant with the truncated LHY was arrhythmic. Interestingly, in this study, *CCA1::LHY*-in-*lhy* null plants assayed at 17°C mimic *cca1-1 lhy-21*, while when assayed at 27°C the same plants act like *cca1-1 lhy-11*. These phenotypes could be related to the amounts of *LHY* present at 17°C versus 27°C.

3.1.3. Discussion:

CCA1 and LHY are main components of the *Arabidopsis* circadian clock. Both proteins exhibit a high protein sequence similarity and are thought to be functionally redundant. Recent work by Gould et al. (2006) demonstrated that CCA1 and LHY play different roles in the temperature compensation of the *Arabidopsis* circadian oscillator: the function of CCA1 is more important at the lower end of the permissive temperature range, while LHY is more important at the higher end, suggesting that CCA1 and LHY are not functionally identical. The current study provides further insight into CCA1 and LHY functional differentiation as well as their roles in buffering the clock against temperature changes. By swapping the native promoters between the *CCA1* and *LHY* genes, and introducing the new constructs into *cca1* and *lhy* null mutants, it was demonstrated that expression of *CCA1* and *LHY* is regulated differently and that this regulation is temperature dependant. Furthermore, it was shown that transcriptional regulation of both genes from their own promoters is of paramount importance in keeping the central oscillator in balance.

First, it was investigated whether the difference in clock buffering capability against temperature changes existed between *cca1-11* and *lhy-21* loss of function mutants. Indeed, even though *cca1-11* and *lhy-21* exhibited similar circadian phenotypes at 17°C, the difference between the two mutants became profound when both were examined at 27°C. *lhy-21* was more temperature sensitive, with more than a 2 h shorter period compared to the wild type, whereas the circadian period of *cca1-11* shortened by only 1 h. This result confirms that CCA1 and LHY play different roles in temperature compensation of the *Arabidopsis* circadian clock, where

functioning LHY is of greater importance at higher temperature than CCA1, which is consistent with the findings of Gould et al. (2006).

It was expected that introduction of *CCA1::CCA1* and *LHY::LHY* transgenes into *cca1-11* and *lhy-21* would rescue their circadian phenotypes and eliminate the difference in temperature compensation capability. Indeed, the majority of plants carrying *CCA1::CCA1* or *LHY::LHY* constructs exhibited a rescued circadian phenotype when examined by the leaf movement technique. However, differences were found between leaf movement period of these *CCA1::CCA1*-in-*cca1* null and *LHY::LHY*-in-*lhy* null transplants. The period of the 3 *CCA1::CCA1*-in-*cca1* null transgenic lines that displayed a rescued phenotype, had circadian period matching the wild type control (Table 3.2; Figure 3.2). In contrast, *lhy* null was not only rescued by the *LHY::LHY* transgene, but 3 out of 6 lines had an approximately 2 h longer period than the control (Table 3.2; Figure 3.3). The period differences between *CCA1::CCA1*-in-*cca1* null and *LHY::LHY*-in-*lhy* null transplants suggested possible alterations in the central clock, for example, variation in *CCA1* and *LHY* transcription.

The expression of *CCA1* and *LHY* genes was examined in both types of plants at dawn after entrainment to a 12:12 L:D photoperiod. In all plants *LHY* was expressed at higher levels than *CCA1*, which is consistent with previous reports (Lu et al., 2009). In *CCA1::CCA1*-in-*cca1* null rescued lines, both *CCA1* and *LHY* were expressed at the levels close to the control (Figure 3.2 inset bars). On the contrary, all *LHY::LHY* -in-*lhy* null rescued lines displayed a reduction of *CCA1* expression by almost half (Figure 3.3). In addition, lines with a long period phenotype exhibited a noticeable increase in *LHY* expression (Figure 3.3). One of the possible explanations for the gene expression differences between various plant lines could be that a

different number of transgenes were incorporated into the genome during the transformation event. Integration of multiple insert copies into a genome happens much more often than integration of a single insert however, mupltiple copies usually land into one locus (De Buck et al., 2004). However, an increased number of transgenes does not usually result in extremely high levels of a transgene expression, as long as the integration is within the same location (reviewed in Butaye et al., 2005). To avoid plants with transgenes in multiple loci, the transformed plants were selected based to the Mendel segregation rule. Therefore, even if both *CCA1::CCA1*-in-*cca1* null and *LHY::LHY*-in-*lhy* null lines carry several copies of a transgene per locus, the effect of such multiple integration is not likely to be the only explanation for differences between the plants. Considering that several lines of the same construct have the same gene expression pattern and similar circadian phenotype, it is safe to assume that differences between *CCA1::CCA1*-in-*cca1* null and *LHY::LHY*-in-*lhy* null lines are caused by actual differences between CCA1 and LHY.

Several *CCA1::CCA1*-in-*cca1* null lines had a circadian phenotype very similar to the wild type, despite a slight variation in *CCA1* and *LHY* mRNA amounts between the transplants and the control (Figure 3.2). The levels of *CCA1* were slightly higher than in the Ws, however, it did not seem to affect circadian rhythms. Increased mRNA levels do not necessarily cause an increase in protein levels which could lead to a phenotypic change, suggesting that CCA1 could be tightly regulated on posttranscriptional or translational levels. On the other hand, it is possible that an increase in *CCA1* would not cause any shift in circadian rhythmicity as long as it is not over-synthesized above a certain maximum dosage. In comparison to the *CCA1::CCA1*-in-*cca1* null lines, *LHY::LHY*-in-*lhy* null plants had higher than Ws

LHY amounts, decreased *CCA1* levels and a long period phenotype (Figure 3.3). As previously mentioned, *LHY* is more highly expressed than *CCA1* (Lu et al., 2009), therefore, it is possible that increased expression of *LHY* is harder to bring down and keep at normal levels, leading to the phenotype alteration. An increase in *LHY*, a core component of the main clock loop, slows down the oscillator resulting in a longer leaf movement period observed in *LHY::LHY*-in-*lhy* null lines. Furthermore, LHY is thought to inhibit transcription of *CCA1* (Wang and Tobin, 1998; Kim et al., 2003), explaining the downregulation of *CCA1* expression in *LHY::LHY*-in-*lhy* null lines.

Interpretation of the results discussed above was obscured by variation in data between lines of the same type. Out of 6 lines used to assess the effect of each transgene, some lines exhibited either a *cca1* or *lhy* null phenotype or acted as overexpressors, suggesting that a higher number of transformed lines should be used to ensure better consistency in results.

Subjection of *CCA1::CCA1*-in-*cca1* null and *LHY::LHY*-in-*lhy* null lines to 27°C revealed, that the temperature increase causes a reduction in *CCA1* and *LHY* expression, with a stronger affect on *LHY*. A decrease in *LHY* mRNA in all *LHY::LHY*-in-*lhy* null lines was coupled with a rise in period variance suggesting a decline in clock precision and a shift towards arrhythmia (Figure 3.8). A time-course of the *LHY* transcription would have been valuable in understanding the temperature dependence of *LHY* expression and if it indeed governs circadian rhythms in *LHY::LHY*-in-*lhy* null lines. Interestingly, in *CCA1::CCA1*-in-*cca1* null lines both *CCA1* and *LHY* levels were also reduced at 27°C, however these transplants were better temperature compensated exhibiting phenotypes similar to those displayed at 17°*C*. Overall, no straightforward correlation between changes in *CCA1* and *LHY*

levels and circadian phenotypes could be established, suggesting that additional elements are involved in buffering the clock against temperature changes. It has recently been shown that genes such as *GI*, *FLC*, *PRR7* and *PRR9* play a role in the *Arabidopsis* temperature response mechanism (Edwards et al., 2005; Salome and McClung, 2005; Gould et al., 2006), however, more research is needed to further investigate the precise function of these elements in temperature compensation and their connection to the circadian clock.

Swapping promoters between *CCA1* and *LHY* genes resulted in considerable differences in circadian phenotype of the transformed plants. Introduction of the *LHY*::*CCA1* almost completely complemented the *cca1* null mutant, resulting in a circadian period different from the wild type by less than 1 h (Table 3.3). In comparison to the *cca1* null, where *CCA1* mRNA could not be detected, *LHY*::*CCA1* lines expressed *CCA1* to levels close to the control in some lines (Figure 3.4). However, *CCA1* expression was lower than the wild type in a few other lines, and overall, levels of *CCA1* and *LHY* varied between individual lines. Nevertheless, such variation did not seem to have much effect on circadian period suggesting that plants are most likely to be adapted to these fluctuations (Figure 3.4, Table 3.3). On the other hand, introduction of *CCA1*::*LHY* did not complement *lhy* null but instead caused a further period decrease (Table 3.3). Analysis of *CCA1* and *LHY* gene expression revealed that transcription of both genes was almost completely suppressed (Figure 3.5). The complete *cca1:lhy* double mutant has a very short period of approximately 19 h (Lu et al., 2009), a phenotype matching the *CCA1*::*LHY*-in-*lhy* null lines. As suggested by Lu *et al.* (2009), absence of *CCA1* and *LHY*, can force other proteins or feedback loops to partially substitute their function and keep the clock oscillating. However, the question remains as to what

caused suppression of *CCA1* and *LHY* in *CCA1*::*LHY*-in-*lhy* null lines, and the possibilities are discussed below.

In plants overexpressing *CCA1* or *LHY* from the CaMV 35S promoter, expression of endogenous *CCA1* and *LHY* is inhibited, indicating that the proteins negatively regulate their own and each other's transcription (Schaffer et al., 1998; Wang and Tobin, 1998). Therefore, suppression of *CCA1* and *LHY* in *CCA1*::*LHY*-in-*lhy* null lines could be caused by an overproduction of *LHY* when driven by the *CCA1* promoter, which feeds back and suppresses expression from the *CCA1* promoter. Indeed, comparison of bioluminescence from *CCA1*:*LUC* and *LHY*:*LUC* showed that images of the *CCA1* lines were much brighter than the ones of the *LHY* (personal observation) suggesting that the *CCA1* promoter could be more active than the *LHY*. Lower activity of the *LHY* promoter could explain a period shorter than the wild type in the *LHY*::*CCA1*-in-*cca1* null lines, where *CCA1* is produced at amounts sufficient to complement the *cca1* null phenotype but not enough for the complete rescue. Furthermore, in the wild type, levels of *LHY* are much higher than of *CCA1* suggesting that the difference between the transcript levels could be due to the differences in mRNA stability between the two genes (Lu *et al.*, 2009). Indeed, *CCA1* mRNA degrades rapidly in the light, while this has not been shown to be the case for *LHY*, where light actually activates *LHY* protein synthesis (Kim et al., 2003; Yakir et al., 2007a). Hence, a more active *CCA1* promoter in combination with *LHY* mRNA less susceptible to degradation could contribute to the suppression of *CCA1* and *LHY* in *CCA1*::*LHY*-in-*lhy* null lines.

Another possibility for the *CCA1* and *LHY* suppression in *CCA1*::*LHY*-in-*lhy* null plants could be a difference in affinity of *CCA1* and *LHY* to each other's promoters. In theory, *LHY* could be highly recruited to the *CCA1* promoter,

especially in the absence of its own promoter. Transcriptional suppression of *CCA1* could be more permanent in the absence of the CCA1 protein whose promoter is occupied by LHY due to the lack of its other substrate (i.e. *LHY* promoter) in *CCA1*::*LHY*-in-*lhy* null lines. Suppression of the promoter by CCA1 and/or LHY could be mediated via homo and hetero dimerization of the two proteins (Lu et al., 2009; Yakir et al., 2009). It is possible that the rate of recruitment to the correct promoter depends on the interaction between each other and formation of the correct complex. Hence, the presence of the *LHY* promoter could be necessary to divert the negative effect from the *CCA1* promoter.

Indeed, despite a few conserved regions present in both *CCA1* and *LHY* promoters, the overall structure of the two is highly divergent (Spensley et al., 2009). The absence of a CHE binding site in the *LHY* promoter provides further support towards difference in *LHY* and *CCA1* transcription regulation (Pruneda-Paz et al., 2009). Availability of an extra binding site for *CCA1* regulatory elements in *CCA1*::*LHY*-in-*lhy* null lines, could cause an imbalance in normal CCA1 and LHY regulation. Indeed, additional copies of the full *CCA1* promoter cause an overexpressor phenotype, including constant high expression of *CCA1* (Ovadia et al., 2010). Interestingly, introduction of the *CCA1* promoter lacking a 5'UTR resulted in almost complete repression of *CCA1* expression. In both cases, transcription of *LHY* continued to oscillate suggesting that regulation of *CCA1* and *LHY* could be completely independent from one another. On the other hand, even though the rhythmicity of *LHY* was unaffected by the addition of the *CCA1* promoter with or without the 5'UTR, there was a reduction in *LHY* amplitude suggesting possible competition for a positive regulator of LHY and CCA1. Overall, it is obvious that

regulation of CCA1 and LHY is complex, and it is clear that it differs significantly between the two genes.

The period of *LHY*::*CCA1*-in-*cca1* null plants was well temperature compensated. At 27°C the period in all lines shortened slightly but it was still comparable with the wild type, indicating that temperature has a similar effect on the transformed lines as it does on the Ws control. Temperature increase caused a considerable reduction in *CCA1* and *LHY* transcripts, however this did not seem to have much effect on the phenotype. Gould et al. (2006) tested the effect of 27°C on *CCA1* and *LHY* expression by frequent sampling of Ws RNA in a continuous light environment. Analysis of *CCA1* and *LHY* mRNA showed that temperature increase had a negative effect on *LHY* transcription, while levels of *CCA1* remained unchanged. In current work, however, RNA was harvested at dawn from plants entrained to 12:12 L:D cycles, therefore, the data could not be directly compared with the Gould et al. (2006) results. It is possible that temperature increase causes a phase shift in gene transcription, and therefore, tissue sampling for mRNA analysis at only 1 time point might not be a good representation of the actual temperature affect on *CCA1* and *LHY* transcription. This hypothesis should be further investigated by carrying out a time-course experiment on the transgenic plants.

Arrhythmia in the *CCA1*::*LHY*-in-*lhy* null lines observed at 27°C was corroborated by an increase in *CCA1* and *LHY* transcript levels, which is the opposite of the effect of temperature in other lines. It is possible that this effect is a result of a complicated interaction between LHY and other circadian clock components. A truly double *cca1:lhy* mutant has a very short period, whilst a *cca1:lhy-11* expresses a truncated LHY protein and is arrhythmic (Lu et al., 2009). As previously suggested, it is possible that in *CCA1*::*LHY*-in-*lhy* null plants, where expression of both genes is

suppressed, other loops can partially substitute CCA1 and LHY function. However, these loops could be not temperature compensated or their temperature range is very narrow, hence abolishing the substitutive role when subjected to temperatures away from the norm. On the other hand, high temperature could interfere with the suppression of the *CCA1* promoter, temporarily releasing it from the inhibition. A minuscule production of *CCA1* and/or *LHY* could be interfering with other members of circadian system aiming to compensate for the loss of CCA1 and LHY. This hypothesis should be further investigated.

3.2. Analysis of the effects of elevated *CCA1* and *LHY* levels on the circadian phenotype

3.2.1. Introduction:

Differences in circadian phenotypes were observed between *cca1*-null plants rescued by *CCA1::CCA1* and *lhy*-nulls rescued by *LHY::LHY*. If arrhythmic lines and lines with a short period phenotype (i.e. non-rescued) are ignored, *CCA1::CCA1* transformants had a circadian period matching the wild type control. On the other hand, *lhy*-null transformants had a period approximately 2 h longer than Ws (Table 3.2). These results indicate that there might be a difference in plant responses to elevated amounts of *CCA1* and *LHY*. To check this speculation, Ws plants were transformed with either *CCA1::CCA1* or *LHY::LHY*, named *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws, respectively.

Circadian phenotype of four homozygous lines from each transformation was assessed using leaf movement, delayed fluorescence and flowering assays. The flowering assay is commonly used as an additional test to identify the proper functioning of the circadian clock, as the circadian clock and the flowering response are interlinked (Yanovsky and Kay, 2002). Mutants that lack functional CCA1 or LHY flower earlier than the wild type, while mutants constitutively overexpressing *CCA1* or *LHY* flower late even when grown under long day conditions, which induce flowering in *Arabidopsis*.

3.2.2. Results:

3.2.2.1. Expression patterns of CCA1 and LHY

To test the degree to which abundance of *CCA1* and *LHY* transcripts was affected by the introduction of *CCA1::CCA1* and *LHY::LHY* constructs into Ws, plant tissue was harvested 1 h after dawn from 10-day-old plants grown under 12:12 L:D conditions. *CCA1* and *LHY* are morning expressed genes with the maximum expression at 1 h after the lights turn on, therefore, harvesting at this time of the day should give a good representation of the synthesized *CCA1* and *LHY* mRNA.

In the wild type, *CCA1* was expressed at lower levels than *LHY*, which is consistent with previous studies (Lu et al., 2009). In *CCA1::CCA1*-in-Ws transformants, *CCA1* was expressed at higher levels than in the wild type. Lines 2 and 10 had a 10% to 30% *CCA1* mRNA increase, respectively, while in lines 4 and 12 *CCA1* mRNA levels increased 2-3 fold. However, in all lines *CCA1* was more abundant than *LHY* (Figure 3.11 A). In addition, elevated expression of *CCA1* correlated with lower levels of *LHY*, which were even lower than in the Ws.

By contrast, in *LHY::LHY*-in-Ws transgenic lines, expression of *LHY* was significantly elevated in comparison to the wild type (Figure 3.11 B). Line 2 had the least increase in *LHY* mRNA abundance when compared with other transformed lines, however, even this line exhibited a 3 fold increase in *LHY*. Line 8 had the highest expression of *LHY*, which was almost 8 times higher than in the wild type (Figure 3.11 B).

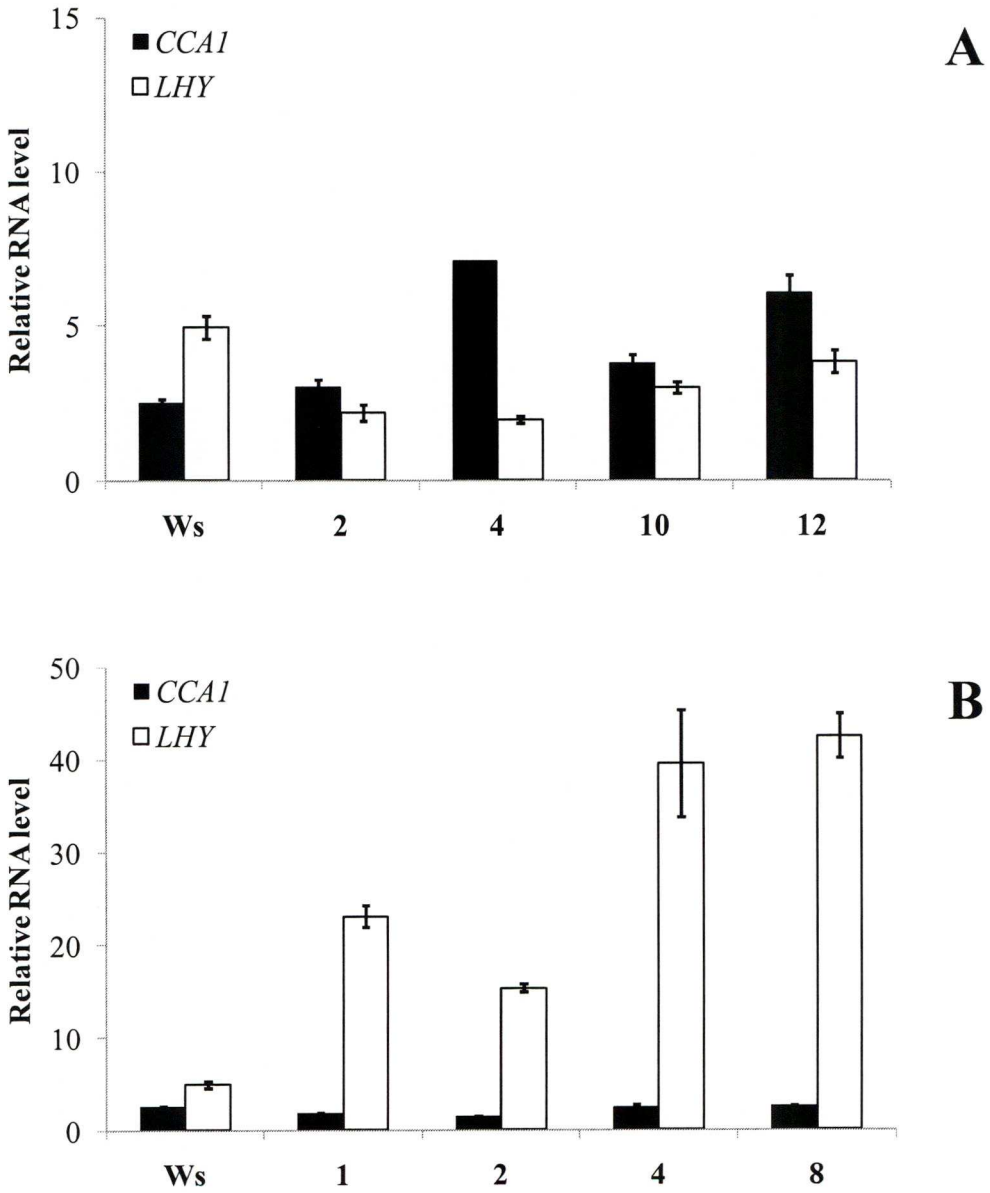


Figure 3.11. Transcript abundance of *CCA1* and *LHY* relative to *UBQ10* (a housekeeping gene) measured in Ws lines transformed with *CCA1::CCA1* (A) or *LHY::LHY* (B).

All plants were grown on MS agar with 3% sucrose under 12:12 L:D for 7 days before the transfer to a highly controlled growth cabinet and grown for additional 3 days at 17°C under 12:12 L:D. Plant tissue was harvested a 1 h after dawn and subjected to qRT-PCR analysis.

In summary, in Ws, abundance of *LHY* mRNA is higher than of *CCA1*. Introduction of *CCA1::CCA1* into Ws caused an increase in *CCA1* levels and a decrease in *LHY*. On the other hand, *LHY::LHY*-in-Ws transformants exhibited a several fold increase in *LHY* expression, however, expression of *CCA1* was only slightly affected.

3.2.2.2. Expression profiles of CCA1 and LHY

To investigate whether additional *CCA1* and *LHY* had an effect on the circadian rhythmicity of their own expression, the accumulation of *CCA1* and *LHY* transcripts over a 24 h period was measured. Two plant lines for each construct were selected. These were grown for 7 days under 12:12 L:D cycles and then transferred to continuous light to free-run for an additional 3 days. Plant tissue of each genotype was harvested starting at subjective dawn and then every 4 h for 24 hours. Abundance of *CCA1* and *LHY* was measured by qRT-PCR.

In Ws the patterns of *CCA1* and *LHY* expression were the same, with the overall levels of *LHY* higher than *CCA1* (Figure 3.12 A). In *CCA1::CCA1*-in-Ws lines the expression profiles of *CCA1* and *LHY* also matched, however, expression of *CCA1* was elevated to the same level as *LHY* (Figure 3.12 B and C). Also, both transcripts had a broader peak of high mRNA abundance in comparison to the Ws, where only 1 well defined peak was observed (Figure 3.12 A).

Expression analysis of *LHY::LHY*-in-Ws lines indicated a great increase in *LHY* abundance. The expression patterns of the 2 examined lines were different, i.e. line 1 produced 2 expression peaks, while line 8 had 1 (Figure 3.13 A and B). In both lines, expression of *CCA1* mimicked the expression pattern of *LHY*.

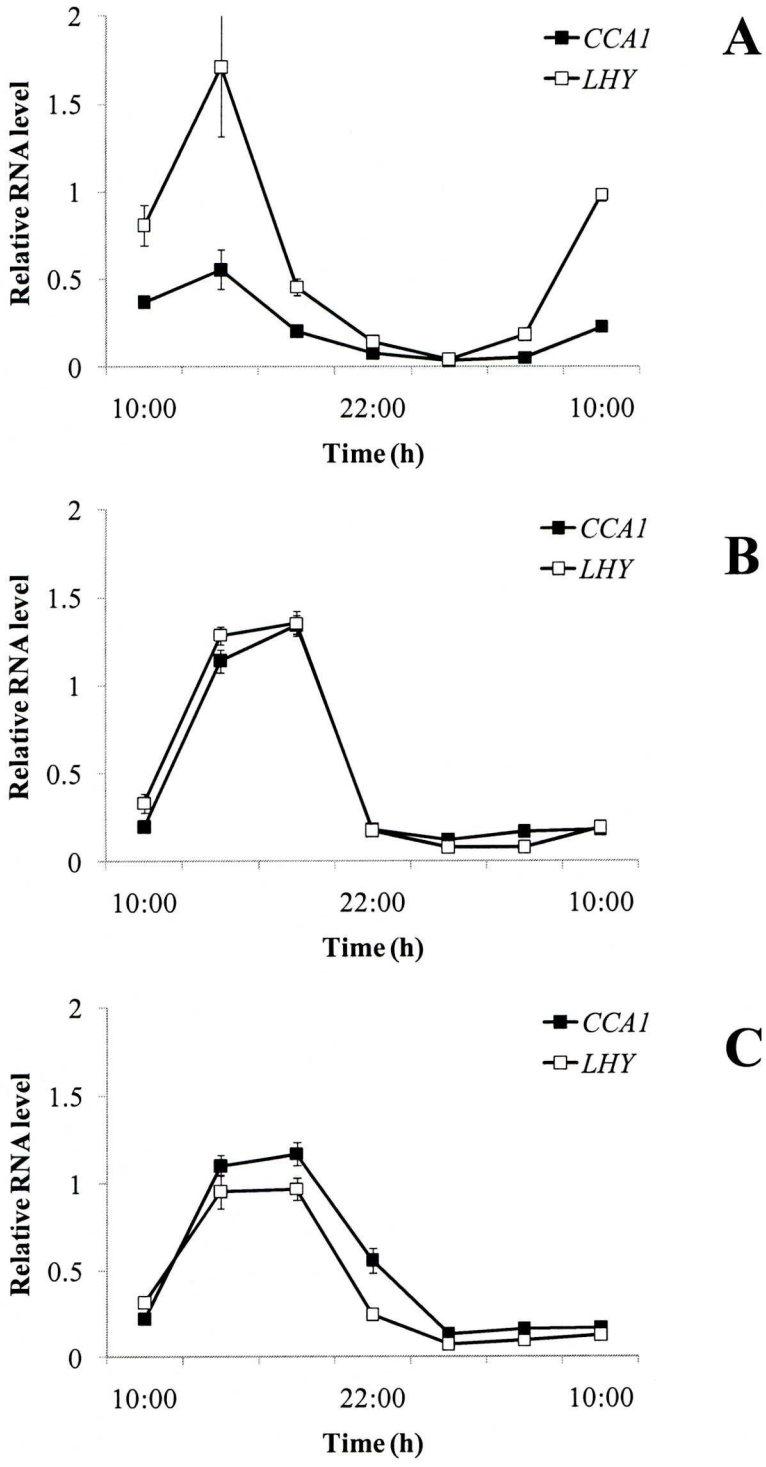


Figure 3.12. *CCA1* and *LHY* expression patterns in Ws (A) and *CCA1::CCA1*-in-Ws lines 2 (B) and 12 (C).

Plants were grown at 22°C 12:12 L:D for 7 days and then transferred to continuous light. Plant tissue was harvested every 4 h for 24 h starting at subjective dawn. The RNA samples were subjected to qRT-PCR and were ran in triplicates.

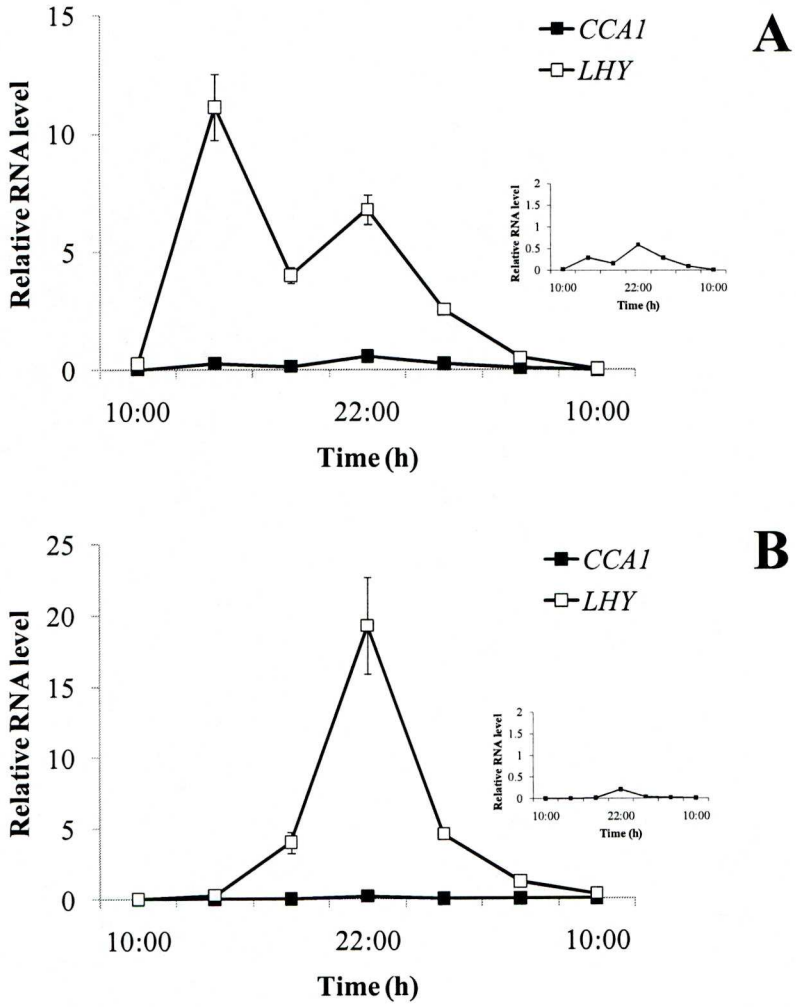


Figure 3.13. *CCA1* and *LHY* expression patterns in *LHY::LHY*-in-Ws lines 1 (A) and 8 (B).

Plants were grown at 22°C 12:12 L:D for 7 days and then transferred to continuous light. Plant tissue was harvested every 4 h for 24 h starting at subjective dawn. The RNA samples were subjected to qRT-PCR and were ran in triplicates. *CCA1* expression for each line was re-plotted on a different Y-axis for better visualisation.

However, levels of *CCA1* mRNA were decreased in line 8 in comparison to the wild type, indicating a potential repression of *CCA1* transcription by LHY, which is produced in very high amounts. On the other hand, repression of CCA1 by LHY is questionable in line 1, where levels of *CCA1* were not much affected and wre even slightly higher than in the wild type. Overall, in all lines expression of *CCA1* and *LHY* mirrored each other and are clearly linked. An increase in *CCA1* in *CCA1::CCA1*-in-Ws lines resulted in a prolonged high expression of *CCA1* and *LHY*, suggesting that both of these genes were highly expressed for a longer period of time. It is also possible that the degradation rate of the mRNA of the two genes was slowed down or the mRNA had become more stable. By contrast, extra *LHY* in *LHY::LHY*-in-Ws transformants resulted in a different pattern of expression of the two genes. These lines expressed *LHY* at very high levels and that was probably the cause for altered pattern of C*CA1* and *LHY* expression.

Comparison of *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws lines suggests that CCA1 and LHY are regulated differently or by a different mechanism. It is obvious that introduction of extra copies of *LHY* into the genome has a larger impact on *CCA1* and *LHY* expression, than extra copies of *CCA1* does. However, in both cases, *CCA1* and *LHY* had a prolonged peak of expression, which would probably cause a longer circadian period in clock outputs. To test this hypothesis, circadian phenotype of *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws lines was assessed.

3.2.2.3. Effect of extra CCA1 and LHY on circadian rhythmicity

To investigate whether increased levels of *CCA1* and *LHY* had an effect on plant circadian phenotype as well as if this effect was different between *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws lines, transformed plants were subjected to leaf movement and delayed fluorescence assays. As predicted, presence of extra

copies of *CCA1* and *LHY* in the *Arabidopsis* genome affected its circadian rhythmicity and this effect was different between *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws plants. Even though *CCA1::CCA1*-in-Ws were able to maintain robust rhythms in leaf movement, these mutants had a 1-2 h increase in their circadian period (Figure 3.14 and Table 3.6). By contrast, *LHY::LHY*-in-Ws lines also maintained robust circadian rhythms, however, the difference in circadian period between the lines and the wild type control increased to 3 h (Figure 3.15 and Table 3.6). Leaf movement data was corroborated by delayed fluorescence data with the only difference of *LHY::LHY*-in-Ws plants having a further increase in the period length than the leaf movement (>30h) (Table 3.7; Suppl. Figure 4 and 5).

In summary, even though overall expression of *CCA1* and *LHY* is much higher in *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws lines than in Ws, leading to the lengthening of the circadian period, the robustness of rhythmicity remains unchanged (Figure 3.14 and 3.15) and indicates a well functioning clock.

3.2.2.4. Effect of extra CCA1 and LHY on flowering time

It has previously been shown that changes in *CCA1* and *LHY* expression affect *Arabidopsis* flowering time (Wang and Tobin, 1998; Mizoguchi et al., 2002). *cca1* and *lhy* null mutants flower early, while plants overexpressing *CCA1* or *LHY* flower very late. Therefore, it is highly possible that introduction of extra copies of *CCA1* and *LHY* into the genome, leading to increased levels of *CCA1* or *LHY*, would have an effect on flowering and cause its delay. To test this hypothesis, the flowering time as well as the vegetative stage of *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws plants were measured. Plants were grown under long days of 16:8 L:D condition, which is known to induce flowering in *Arabidopsis* (reviewed in Devlin and Kay, 2000).

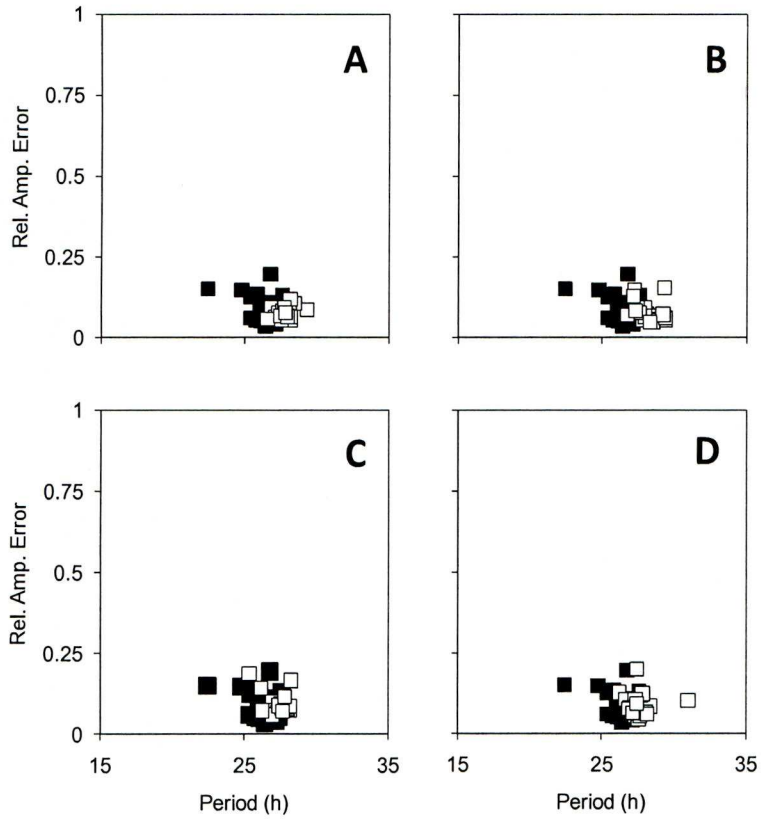


Figure 3.14. Analysis of leaf movement in *CCA1::CCA1*-in-Ws transgenic lines. Transformation of Ws with *CCA1::CCA1* causes a slight lengthening of the circadian period.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, wild type Ws (n=20), open squares, transgenic line (n=20). Individual transgenic lines 2, 4, 10 and 12 are plotted on graphs A to D respectively.

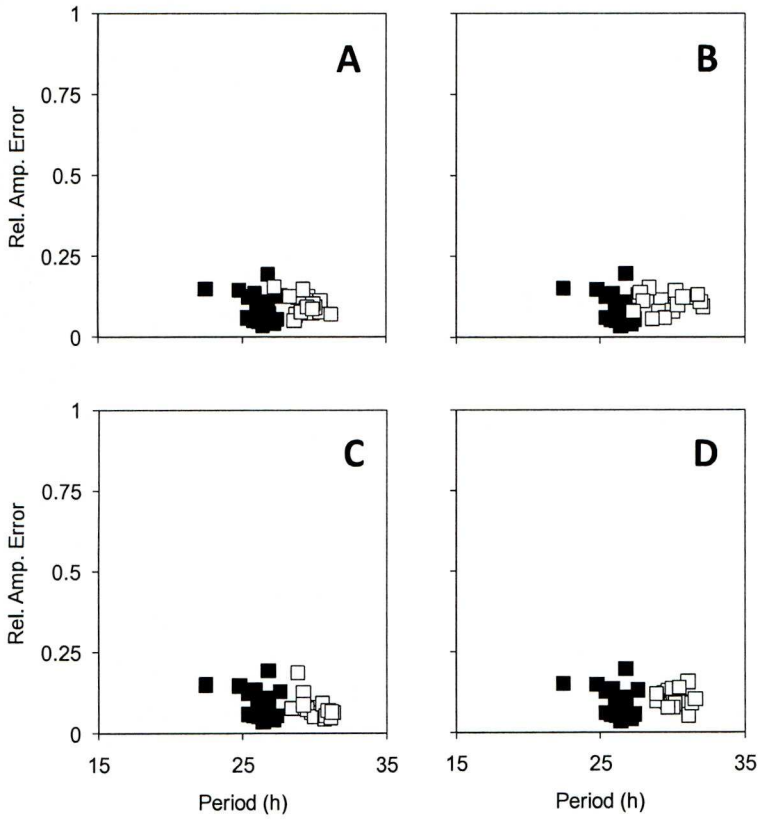


Figure 3.15. Analysis of leaf movement in *LHY::LHY*-in-Ws transgenic lines. Transformation of Ws with *LHY::LHY* causes a considerable lengthening of the circadian period.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, wild type Ws (n=20), open squares, transgenic line (n=20). Individual transgenic lines 1, 2, 4 and 8 are plotted on graphs A to D respectively.

Table 3.6. Leaf movement period estimates for *CCA1::CCA1* and *LHY::LHY-* in-Ws lines

Name	Line Number	Period (h)	SD	n
Ws		26.21	0.43	20
CCA1::CCA1				
	L.2	27.6	0.3	19
	L.4	28.3	0.3	19
	L.10	27.5	0.4	20
	L.12	27.3	0.3	20
LHY::LHY				
	L.1	29.3	0.5	20
	L.2	29.0	1.0	19
	L.4	29.9	0.5	18
	L.8	29.6	0.5	19

Table 3.7. Delayed fluorescence period estimates for *CCA1::CCA1* and *LHY::LHY-* in-Ws lines

Name	Line Number	Period (h)	SD	n
Ws		25.2	0.5	8
CCA1::CCA1				
	L.2	27.6	0.4	8
	L.4	28.0	0.9	8
	L.10	27.2	0.3	8
	L.12	27.7	0.6	8
LHY::LHY				
	L.1	31.3	0.7	8
	L.2	32.2	0.6	8
	L.4	32.1	0.7	8
	L.8	29.4	0.9	8

As predicted, all transgenic lines flowered later than the Ws control. The delay ranged from 3 days in *CCA1::CCA1*-in-Ws line 2 to 12 days in *LHY::LHY*-in-Ws line 8 (Figure 3.16). The vegetative development of the transgenic plants, indicated by the number of rosette leaves, was also affected, with all mutants flowering at the later developmental stage.

These results clearly demonstrate that levels of *CCA1* or *LHY* effect the flowering time in *Arabidopsis*. However, interestingly, even though a substantial difference between *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws lines was observed in their circadian periodicity, the differential effect of CCA1 or LHY on flowering was not as obvious.

3.2.2.5. Effect of extra CCA1 and LHY on temperature compensation

To investigate whether elevated levels of *CCA1* and *LHY* affected the temperature compensation of the circadian clock, circadian rhythms of leaf movement at 17°C and 27°C were compared. Seedlings of *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws transformants were grown at 17°C under 12:12 L:D conditions and after 10 days were transferred to either 17°C or 27°C and continuous light, where their free-running periods were measured.

Figures 3.17 and 3.18 show that temperature increase did not have a pronounced effect on the leaf movement as all lines were able to maintain robust circadian rhythms, indicating that the clock was still well temperature compensated. The circadian period of *CCA1::CCA1*-in-Ws mutants was not at all affected by the temperature (Figure 3.17). Interestingly, *LHY::LHY*-in-Ws circadian phenotype was temperature-dependent (Figure 3.18). At 27°C all lines exhibited a further circadian period lengthening in comparison to the already long-period phenotype at 17°C.

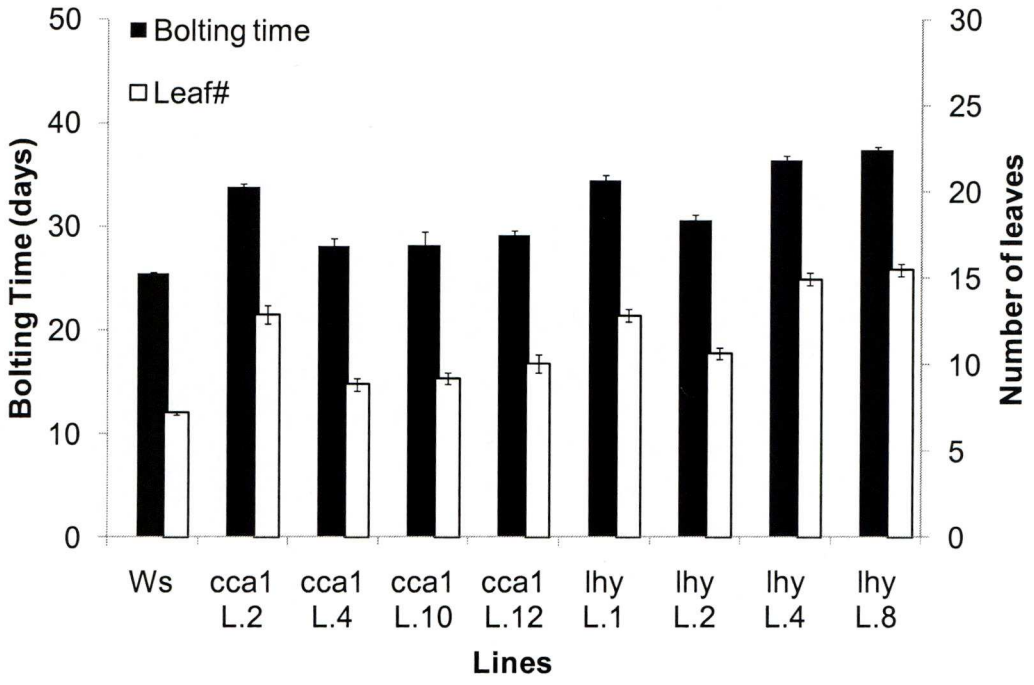


Figure 3.16. Flowering time for *CCA1::CCA1* and *LHY::LHY*-in-Ws transgenic lines.

Bolting time and number of rosette leaves (mean +/- SE) for wild types Ws and transgenic *Arabidopsis* lines are presented. Plants were grown at 22°C under 16:8 L:D conditions.

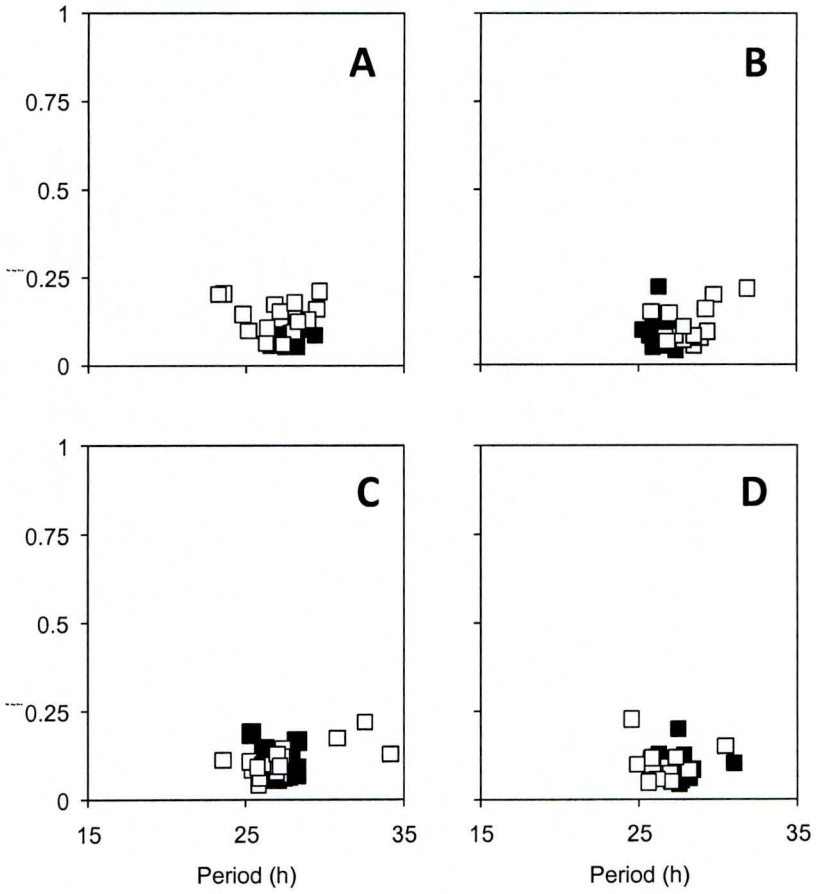


Figure 3.17. Temperature compensation of leaf movement for *CCA1::CCA1*-in-Ws transgenic lines. Introduction of additional *CCA1* to Ws plants does not affect circadian temperature compensation.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C or 27°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, 17°C (n=20), open squares, 27°C (n=20). Individual transgenic lines 1, 2, 4 and 8 are plotted on graphs A to D respectively.

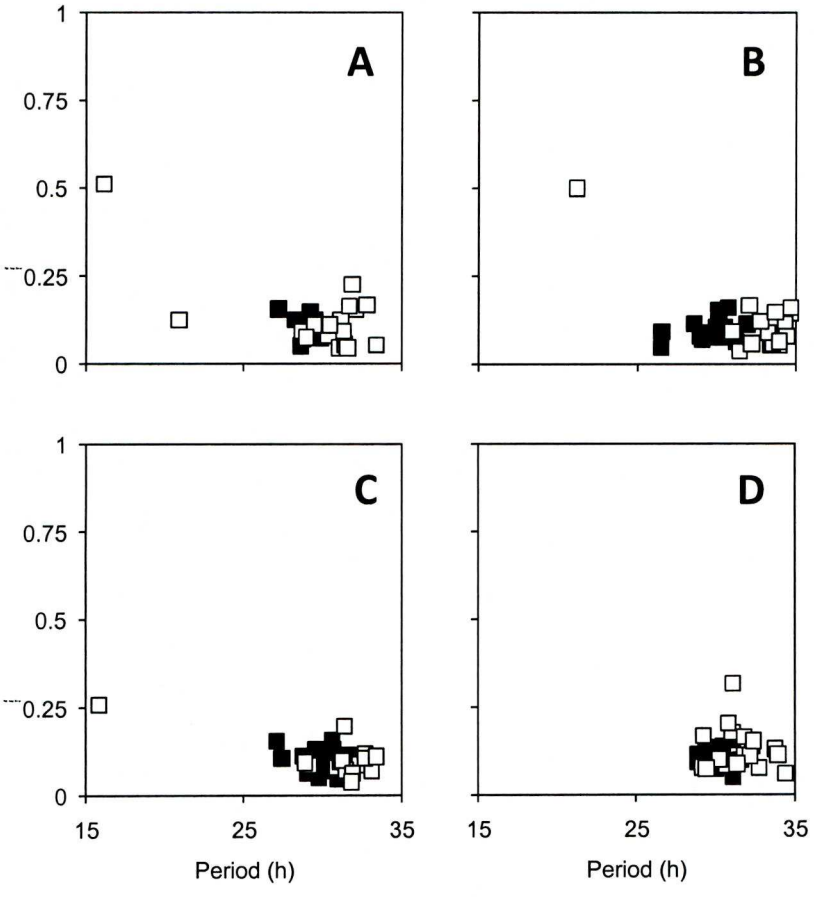


Figure 3.18. Temperature compensation of leaf movement for *LHY::LHY*-in-Ws transgenic lines. Introduction of additional *LHY* to Ws plants slightly affects their ability to buffer the clock against the temperature change.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 10 days before the transfer to 17°C or 27°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, 17°C (n=20), open squares, 27°C (n=20). Individual transgenic lines 1, 2, 4 and 8 are plotted on graphs A to D respectively.

These results clearly suggest that CCA1 and LHY have temperature-dependant functions. No temperature effect was observed in *CCA1::CCA1*-in-Ws, however, the same temperature increase caused period lengthening in *LHY::LHY*-in-Ws lines. It is possible that temperature either promoted transcription of *LHY*, or stabilized its mRNA. Analysis of *CCA1* and *LHY* expression profiles under free-running conditions at 27°C would give a better understanding of whether transcript abundance is temperature regulated and whether circadian period lengthening at 27°C is a result of the period lengthening in *CCA1* and *LHY* expression. Unfortunately, this data is not available at the moment.

3.2.3. Discussion:

CCA1 and LHY are MYB-like transcription factors possessing activator (e.g. expression of CAB) or repressor (e.g. TOC1) functions (Harmer and Kay, 2005). Overexpression of either of the genes from the 35S CV promoter causes arrhythmia in circadian oscillations, a late flowering phenotype, as well as repression of endogenous *CCA1* and *LHY* (Schaffer et al., 1998; Wang and Tobin, 1998). Both overexpressors have the same phenotype suggesting that CCA1 and LHY have redundant roles. Here, the effect of the increased gene dosage of *CCA1* and *LHY* was investigated. Addition of extra *CCA1* and *LHY* copies onto the Ws background resulted in differences in overt characteristics between *CCA1*::*CCA1*-in-Ws and *LHY*::*LHY*-in-Ws lines. Introduction of *CCA1::CCA1* into the Ws brought expression of *CCA1* to the levels similar to that of *LHY* (Figure 3.12). The amount of *CCA1* in these lines was higher than the ones measured in the *CCA1* overexpressor, where *CCA1* is expressed at levels equivalent to the maximum wild-type levels (Wang and Tobin, 1998). In *LHY::LHY*-in-Ws lines, on the other hand, addition of extra *LHY* genes caused an enormous overproduction of *LHY* mRNA, in some cases reaching up to an 8 fold increase (Figure 3.11). The differences between the degree of *CCA1* and *LHY* expression were not too surprising, as normally *LHY* is expressed at much higher levels than *CCA1* (Lu *et al.*, 2009), explaining a much greater increase in *LHY* in *LHY::LHY*-in-Ws lines than *CCA1* in the *CCA1::CCA1*-in-Ws plants. It was surprising however, that despite a multi-fold increase in *CCA1* and *LHY* transcript in *CCA1*::*CCA1*-in-Ws and *LHY*::*LHY*-in-Ws lines respectively, both types of plants retained their circadian rhythmicity.

CCA1 and LHY inhibit each others' transcription (Wang and Tobin, 1998; Kim et al., 2003), however current data suggests that such interaction is more

complex. When *CCA1* and *LHY* transcript abundance was measured from plant tissue collected at dawn after 12:12 L:D entrainment, it appeared that expression of *LHY* was partially down-regulated, as expected. However, when *CCA1* and *LHY* transcription patterns were examined over the course of 24 h, the inhibition effect was absent. At subjective dawn the levels of *LHY* were indeed lower than levels *CCA1* as observed during the 1 time-point harvest, however, it is obvious that this type of data does not represent the true picture of the circadian oscillator functioning or provide evidence for CCA1 acting as a repressor.

Addition of extra *LHY* caused an immense increase in *LHY* expression. However, it only resulted in a slight down-regulation of the *CCA1* in one out of two lines, where the maximum *LHY* amount was 10 times higher than of the control. Highly elevated levels of LHY have probably overcome the mechanism regulating activation/repression of *CCA1* expression, especially if the affinity to the *CCA1* promoter is high. While directly repressing one another, CCA1 and LHY may also somehow interact with other negative regulatory elements and thus promote their own expression. It has recently been shown that several members of pseudo response regulator (PRR) family act to repress *CCA1* and *LHY* (Nakamichi et al., 2005). PRRs lack the ability to directly bind to promoters (Nakamichi et al., 2005; Nakamichi et al., 2010), which suggests that other proteins could be recruited for this interaction. While CHE protein is highly likely to be involved in PRRs-CCA1 interaction and repression of *CCA1*, other proteins e.g. TOC1 have been proposed to divert their inhibitory effect (Nakamichi et al., 2010). The absence of CHE association with the *LHY* promoter implies variation in regulation between CCA1 and LHY and a possibility of another, *LHY* specific, regulatory mechanism (Pruneda-Paz et al., 2009). Nevertheless, it is obvious that transcription of both genes is

interconnected as the patters of their expression mimic one another regardless of the large changes in their amounts.

A several fold increase in *CCA1* or *LHY* mRNA did not result in a severe disruption in circadian clock outputs, but only caused a slight increase in the period length. Increased dosage of TOC1 has been reported to lengthen the free-running period in *Arabidopsis* providing support to the current observations (Mas et al., 2003a). Interestingly, it appears that *Arabidopsis* is highly adapted to fluctuations in amounts of the *CCA1* and *LHY* mRNA. Such fluctuations could be necessary and beneficial for plants when under certain environmental conditions. This hypothesis should be further investigated.

The introduction of extra *LHY* copies had a more profound effect on the period of the measured circadian rhythms than *CCA1* did, resulting in a longer circadian period in the *LHY*::*LHY*-in-Ws plants. This is more likely to be due to the much higher levels of *LHY* expression. It is possible, that if the abundance of *CCA1* mRNA was raised to the same level as of *LHY*, *CCA1*::*CCA1*-in-Ws lines would have the same degree of the period lengthening.

Overall, plants with extra copies of the full genes did not abolish their circadian rhythmicity in the way *CCA1* and *LHY* overexpressors did. In both overexpressors *CCA1* and *LHY* transcription is driven from a constitutively expressed promoter (Schaffer et al., 1998; Wang and Tobin, 1998). The absence of rhythmicity in *CCA1/LHY* expression causes a severe disruption in the circadian system and other phenotypic changes i.e. hypocotyl length and flowering. However, introduction of extra *CCA1* and *LHY* gene copies did not disrupt the clock, even though their transcription levels were greatly elevated. Current results imply that

rhythmicity in *CCA1* and *LHY* expression drives circadian rhythms within a plant, and this is highly dependent on the correct promoter.

Interestingly, addition of extra copies of *CCA1* promoter has been shown to cause a complete abolishment in circadian outputs, including late flowering (Ovadia et al., 2010). Late flowering phenotype is also observed in plants overexpressing *CCA1* or *LHY* (Schaffer et al., 1998; Wang and Tobin, 1998). Here, introduction of extra *CCA1* or *LHY* copies caused a flowering delay in the new transplants, supporting the close link between the circadian clock and *Arabidopsis* flowering pathways. However, despite a several fold difference in the *CCA1* and *LHY* expression between *CCA1::CCA1*-in-Ws and *LHY::LHY*-in-Ws lines and the Ws control, the difference in flowering time was only a few days and in some cases overlapped. Therefore, even though the circadian clock is tightly linked with flowering (Yanovsky and Kay, 2002), the effect of *CCA1* and *LHY* is not directly proportional and is probably strongly counterbalanced with other proteins.

Circadian phenotype of both *CCA1::CCA1* and *LHY::LHY* in Ws lines was well temperature compensated and retained rhythmicity at 27°C. Despite this, plants with extra *LHY* exhibited a further period lengthening in comparison to the lines carrying extra copies of *CCA1* where circadian period remained unchanged. Unfortunately, the temperature effect on *CCA1* and *LHY* transcription in the transgenic lines is absent from the current study and would be very useful in understanding the temperature effect on plants with elevated *LHY*. Analysis of the *CCA1* and *LHY* transcript at 27°C from the Ws, together with the *CCA1* and *LHY* time course data from Gould et al. (2006) suggest that temperature has an inhibiting effect on the *LHY* transcription. If this is true, one should expect a period shortening, but not lengthening in plants subjected to the 27°C. Possibly a decrease in *LHY*

under increasing temperatures is required to keep circadian system balanced, however in *LHY*::*LHY*-in-Ws lines the amount of *LHY* is still above the threshold and therefore, the temperature dependant change in phenotype is observed. On the other hand, the overall dosage of *CCA1* and *LHY* and its imbalance could be of significance. Discovery of the CCA1 and LHY homo and hetero-dimerization provides one possible hypothesis for such imbalance, as addition of extra *CCA1* and *LHY* gene copies could change the ratio in CCA1 and LHY homo and hetero-dimers (Lu et al., 2009; Yakir et al., 2009). In addition, these homo- and hetero-dimers could potentially have different functional properties e.g. DNA binding or protein degradation and consequently be a cause in variation between *CCA1*::*CCA1*-in-Ws and *LHY*::*LHY*-in-Ws phenotypes (Lu et al., 2009). Furthermore, LHY and CCA1 can network differently with other proteins which help buffering the clock against temperature changes. One of these proteins could be GI, whose interaction with LHY and CCA1 is selective and temperature-dependant (Gould et al., 2006). Nevertheless, both proteins are found in the same protein complex and working together (Lu et al., 2009), hence, the balance between the two is highly likely to be important.

Chapter 4 – Natural variation in temperature compensation in *Arabidopsis thaliana*

4.1. Introduction:

Arabidopsis thaliana has been used as a model organism in plant biology since the 1950s. It is small, easy to grow and has a relatively short life cycle, with a new generation of seeds available in just under a 2 month period (Pigliucci, 1998). *Arabidopsis* plants are mainly self-pollinated, resulting in highly inbred, predominantly homozygous lines. It has a relatively small genome, divided over 5 chromosomes, the full sequencing of which made it an even more attractive organism to work with (Rhee et al., 2003). Transformation of *Arabidopsis* via the *Agrobacterium* "floral dip" technique (Davis et al., 2009) has proved to be very efficient and led to the creation of large collections of knock-out and overexpressor mutants available for plant genetic studies (reviewed in Windels et al., 2008). Transformation of other plants, including crops, is not as advanced and, thus, more labour intensive and time consuming (Gelvin, 2003). However, many physiological processes in plants have been found to be highly conserved, therefore, the knowledge acquired from *Arabidopsis* research can be readily exploited and applied to other plant systems (Reymond et al., 2007).

As a common weed, *Arabidopsis* has a broad geographical distribution and is readily found throughout Europe as well as in parts of Asia, Africa and North America (Alonso-Blanco and Koornneef, 2000). The range of habitats preferred by *Arabidopsis* is very wide, resulting in a large phenotypic variation between different ecotypes of *Arabidopsis* plants, otherwise known as accessions (Pigliucci, 1998; Koornneef et al., 2004). Accessions originating from different habitats are also

expected to carry substantial genetic variation, reflecting their adaptation to the specific environments they originate from. In *Arabidopsis*, natural variation has been reported for a number of traits including plant size and morphology, seed dormancy, flowering, and responses to abiotic and biotic stress (Alonso-Blanco and Koornneef, 2000). Also, because *Arabidopsis* is predominantly self-pollinated and usually homozygous, it provides an excellent model to study natural variation and mechanisms underlying environmental adaptation (reviewed in Mitchell-Olds and Schmitt, 2006).

Observed phenotype is a result of interaction between the genotype and the environment. Accordingly, genetic variation often underlies phenotypic variation of traits present in accessions collected from different environments (reviewed in Reymond et al., 2007). Usually, a single trait is controlled by a combination of genes from multiple loci and the variation observed between accessions is thought to be a result of both variable degrees of loci contribution towards the trait expression, as well as gene allelic variation present between plant lines (Shindo et al., 2007). Using genetic variation, a quantitative-trait loci (QTL) analysis has been developed which identifies regions on chromosomes that contribute to differences in phenotypic expression between accessions (Koornneef et al., 2004). By altering growth conditions it is possible to map QTLs and consequently gene candidates, which control specific condition-dependent traits of interest. A number of QTLs responsible for trait variation have been discovered in this way, including natural variation in flowering and circadian period (Swarup et al., 1999; Michael et al., 2003a; Koornneef et al., 2004; Edwards et al., 2005; Darrah et al., 2006).

A circadian clock, synchronized with the environment, has been shown to significantly increase the survival and productivity of a plant (Green et al., 2002;

Dodd et al., 2005; Gardner et al., 2006; Gould et al., 2006). *Arabidopsis* mutants with a disrupted clock were less fit and produced less viable seeds when grown under day:night conditions not matching their circadian phenotype (Green et al., 2002). Thus, a functional clock adapted to a specific day:night conditions and temperature environment could substantially benefit a plant resulting in enhanced growth performance and yield (Dodd et al., 2005). Circadian rhythms are rhythms that oscillate with a period of approximately 24 h. Analysis of *Arabidopsis* leaf movement under free-running conditions revealed that circadian periods range from 22 h to 28 h depending on accession (Michael et al., 2003a), indicating that natural variation exists in circadian periodicity and is likely to be indicative of adaptation to the specific day:night environment. Interestingly, a cross between Landsberg erecta and Columbia accessions, with circadian leaf movement periods of 23 h, resulted in a population with circadian periods varying from 20 to 25 h, suggesting that 23 h oscillations is an end result of a well balanced relationship between period-lengthening and period-shortening alleles and loci (Swarup et al., 1999). Michael et al. (2003a) found a positive correlation between the circadian period of accessions and their geographical latitude, and the day length associated with it. In addition, analysis of Ler-Col recombinant inbred lines revealed that members of the pseudo-response regulator (PRR) family might be involved in variation of circadian period (Michael *et al.*, 2003a). PRR3 together with SRR1 (*SIGNALING IN RED LIGHT REDUCED 1*) have been selected as potential candidates that may be involved in natural variation in phase phenotype, and were identified via analysis of a circadian *CAB2::LUC* reporter gene in recombinant inbred lines derived from a cross between Landsberg and Cape Verde Island accessions (Darrah et al., 2006).

Temperature compensation of period is one of the main characteristics of circadian rhythms (Pittendrigh, 1954). Variation in temperature compensation between *Arabidopsis* accessions has been described by Edwards et al. (2005). Using leaf movement assay, it was observed that some accessions were better temperature compensated, i.e. maintained a constant period, while others exhibited period shortening or lengthening in response to changes in temperature. However, no strong correlation was found between temperature-dependent period response and an accession's geographical location, with the exception of altitude, where accessions from high altitude exhibited a shorter period when subjected to 27°C treatment. A very week correlation between circadian period at 22°C and longitude was also detected, but it was absent at high or low temperatures. The natural variation in temperature compensation of the clock could reflect the local temperature and light environment and not the geographical position a particular accession is adapted to, and suggests the possibility of variation in circadian system controls resulting in a shorter or longer period when under various conditions. Often, detailed information on the habitat where accession seeds have been obtained is very limited, thus obscuring determination of a possible correlation between plant fitness and their environment (Mitchell-Olds and Schmitt, 2006). Seasonal factors e.g. seed storage, maternal photoperiod, seed dispersal and germination time, can also strongly influence subsequent plant generation and affect interpretation of results obtained from the laboratory experiments (Munir et al., 2001; Donohue et al., 2005; Penfield and Hall, 2009). This information is also difficult to obtain from a seed providing organization. In addition, a combination of factors, rather than just one, might be underlying the variation found in any studied parameter (Callahan and Pigliucci,

2002) and this, therefore, makes the establishment of a linear correlation between a plant phenotype and an environmental parameter improbable.

Analysis of temperature-dependent circadian period QTL identified potential candidates involved in temperature compensation (Edwards et al., 2005). *GI* was amongst the candidates and was later proven to have a significant role in the *Arabidopsis* temperature response (Gould et al., 2006) (see introduction for more detailed description). However, data indicated that *GI* could not be solely responsible for temperature compensation and must be working in combination with other genes, potentially including core clock components such as *LHY* (Edwards et al., 2005; Gould et al., 2006).

If a functional circadian clock is beneficial for plant performance, it should be of adaptive value to be able to track exact time regardless of temperature. Studies discussed previously have shown that considerable variation is present in circadian parameters and temperature compensation (Swarup et al., 1999; Michael et al., 2003; Edwards et al., 2005). This study aimed to provide further insight on natural variation in temperature compensation of the circadian clock using phenotypic and molecular clock outputs. Leaf movement assay was used to assess changes in circadian rhythm at different temperatures, while expression of the firefly luciferase reporter gene from *CCA1* and *LHY* promoters was used to monitor the performance of the circadian clock. The reasoning behind choosing *CCA1* and *LHY* but not *TOC1* for the clock assessment was a recent study by Gould et al. (2006), which shows differentiation of CCA1 and LHY roles when subjected to different temperatures. If CCA1 and LHY do play a role in temperature compensation, then natural variation in *CCA1* and *LHY* expression is highly likely.

Analysis of leaf movement and *CCA1* and *LHY* expression at 17°C versus 27°C revealed considerable variation in temperature compensation in all 3 circadian outputs. Secondly, increased temperature caused shortening of the *CCA1* and *LHY* expression period in all plants, however, the direction and the degree of leaf movement period change (if any) was highly accession specific. Further temperature increase resulted in a radical change in the circadian clock mechanism, where *LHY* became arrhythmic while *CCA1* continued to oscillate in a number of accessions. It was revealed that elevated temperature not only affects circadian clock mechanism, but also causes uncoupling of the circadian loops. The degree of uncoupling varied between accessions and could be connected to the accession specific temperature range permissive for circadian rhythmicity. Furthermore, it was shown that circadian temperature compensation is linked to growth performance, where the growth of plants possessing a better buffered clock is less temperature dependant.

4.2. Results:

4.2.1. Geographical distribution of accessions used in the study

A collection of seeds from 18 *Arabidopsis* accessions were used for analysis of natural variation in temperature compensation of the circadian clock. The majority of accessions originated from Europe, however a few came from other parts of the world, including Canada, Japan and the Cape Verde Islands (Figure 4.1, Table 2.2 in the Materials and Methods section). Accessions were selected based upon seed availability in the lab, and additional accessions Je54, Or-0, Wc-1, Dog-5 and Phw-19 were purchased from TAIR. These extra accessions were chosen as they had been previously used in a temperature sensitive/temperature tolerant assay where chlorophyll accumulation in leaves was measured before and after heat treatment at 38°C (J. Burke, pers. commun.). Accessions Dog-5, Wc-1 and Phw-19 were heat tolerant, while Je54 and Or-0 were heat sensitive lines.

4.2.2. Leaf movement

4.2.2.1. Natural variation in temperature compensation using leaf movement assay

To investigate variation in temperature compensation capacity amongst accessions, their circadian rhythms were first assayed by leaf movement at 17°C and 27°C. Seedlings were grown under 12:12 L:D at 17°C and after 10 days transferred to continuous light and appropriate temperature (17°C or 27°C), where their periods were measured. At 27°C a general shift towards period shortening was observed, with more accessions exhibiting a rhythm of a 22-23 h periodicity, in comparison to 25-27 h at 17°C (Figure 4.2).

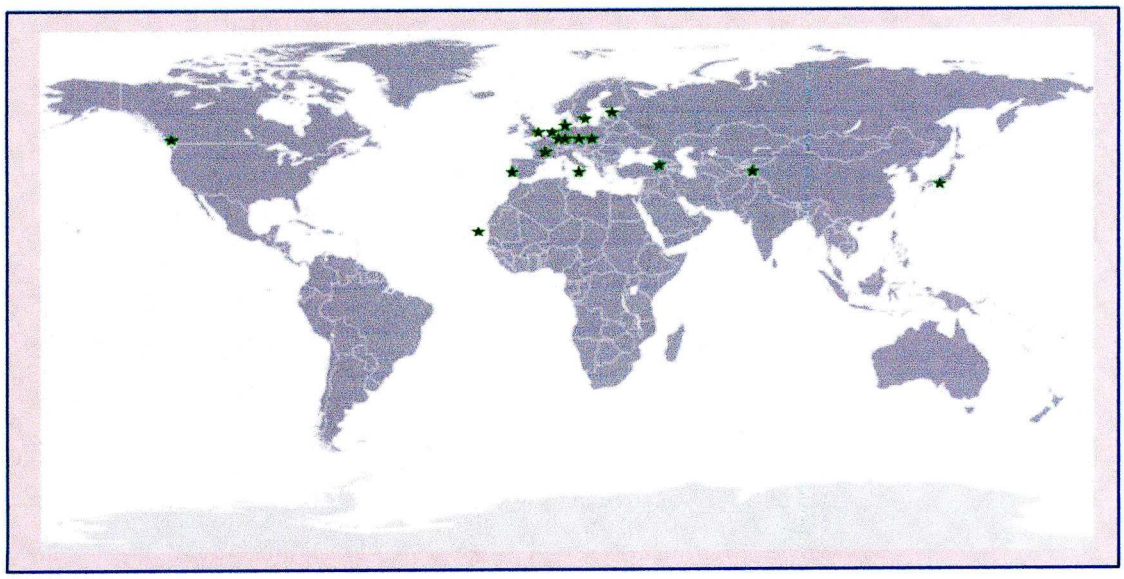


Figure 4.1. Map illustrating *Arabidopsis thaliana* accessions used in this study.

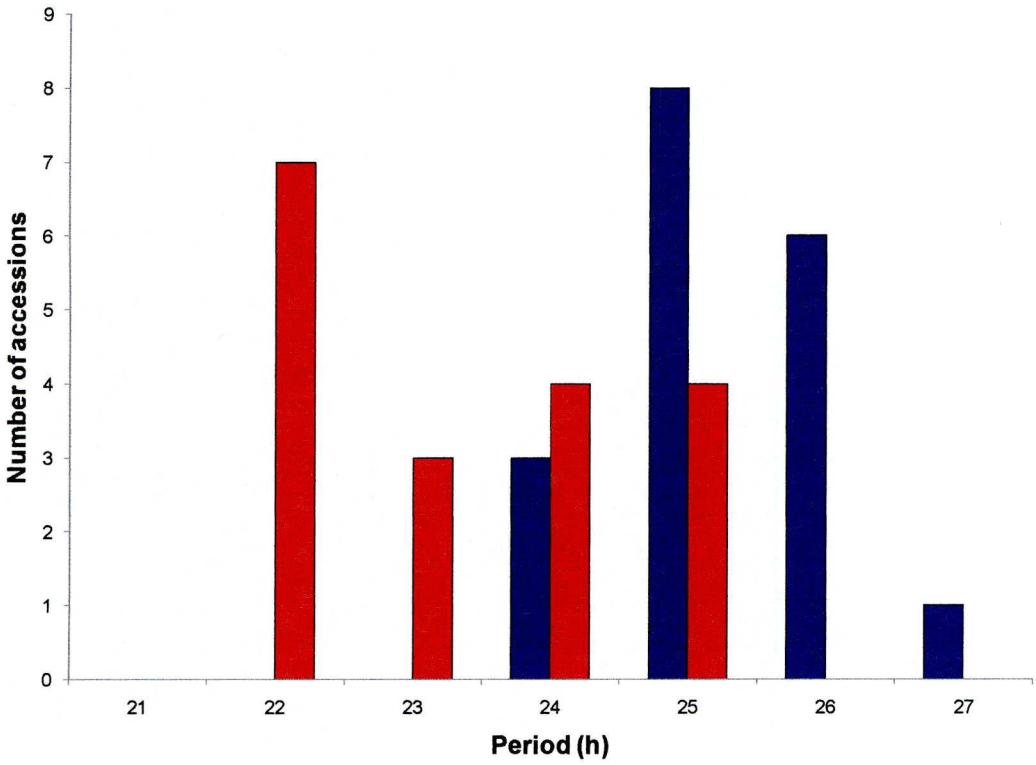


Figure 4.2. Leaf movement period distribution measured in 18 *Arabidopsis* accessions at 17°C (blue bars) and 27°C (red bars).

These results are consistent with observations made by Edwards *et al.* (2005), where accessions showed an overall period decrease when assayed at 27°C compared to 22°C. Relative amplitude error (RAE) was also calculated for each accession and is presented in Table 4.1 together with period estimates. RAE evaluates period robustness and varies from 0 (robust rhythm) to 1 (no rhythm) (Dowson-Day and Millar, 1999). Figure 4.3 illustrates period values from all accessions plotted against their RAE. In general, a slight increase in RAE values was observed at 27°C, shown by the spread of data points on the graph, but, overall, leaf movement was well temperature compensated and largely overlapped with the 17°C data points.

The frequency histogram (Figure 4.2) indicated that accessions exhibited a period shortening when subjected to high temperature, however, accession-specific variation in this response was still observed. Depending on the magnitude of the period response to elevated temperature, accessions were divided into 3 categories: 1 – no change in period between 17°C and 27°C, 2 – less than 3 h decrease and 3 – more than 3 h decrease (Figure 4.4). The first category was comprised of only 4 accessions (C24, Ct-1, Je54 and Kyo), whose circadian periods remained unchanged with the temperature increase. However, unchanged period average did not always signify a completely temperature buffered clock. For example, when period estimates were plotted against RAE values (Figure 4.5, Kyo), it was noted that even though the average period for Kyo was statistically the same between 17°C and 27°C, the range of RAE data considerably expanded. This is shown by the spread of data points along the X-axis at 27°C on the RAE plot in comparison to the tightly clustered points at 17°C, indicating that the circadian periods of individual lines within the accession did not all match and, therefore, the precision of the clock had been affected.

Table 4.1. Natural variation in leaf movement period (±SE) and RAE (real amplitude error) at 17°C and 27°C. SE – standard error.

	17°C			27°C		
Accession	Period (h)	SE	RAE	Period (h)	SE	RAE
An1	25.81	0.17	0.08	22.12	0.26	0.15
C24	24.87	0.10	0.08	24.78	0.48	0.08
Col-0	25.30	0.12	0.08	24.13	0.44	0.16
Ct-1	25.19	0.11	0.06	25.24	0.22	0.10
Cvi	25.95	0.08	0.10	22.87	0.42	0.11
Dog-5	25.35	0.09	0.10	24.61	0.19	0.09
Eri	25.00	0.05	0.06	22.85	0.30	0.12
Est	26.60	0.25	0.10	22.80	0.13	0.11
Fei-0	26.98	0.10	0.06	23.79	0.20	0.11
Je54	25.49	0.15	0.09	25.41	0.09	0.11
Kyo	25.83	0.14	0.09	25.24	0.51	0.17
Ler	24.89	0.14	0.08	22.79	0.08	0.05
Or-0	26.18	0.08	0.05	22.83	0.20	0.08
Phw-19	26.04	0.09	0.10	23.66	0.31	0.11
Sha	24.52	0.25	0.08	22.86	0.12	0.08
Van-0	27.51	0.18	0.11	25.82	0.47	0.06
Wc-1	26.20	0.12	0.10	23.96	0.53	0.17
Ws	26.21	0.09	0.06	24.87	0.39	0.12

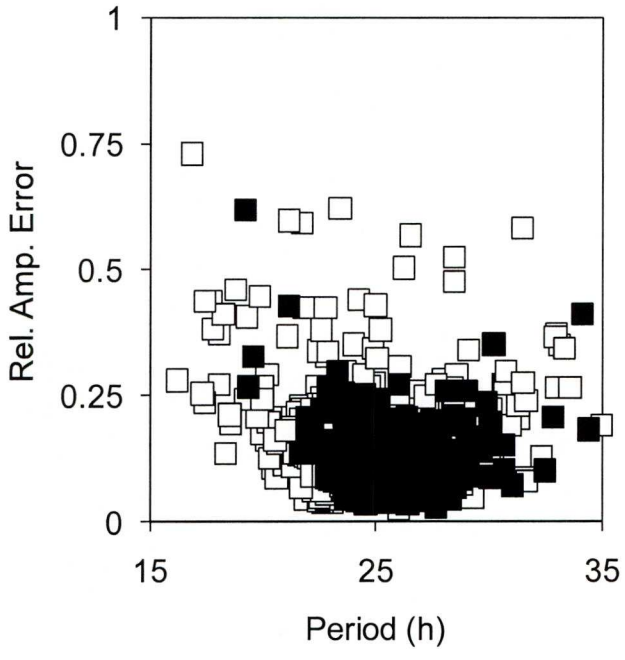


Figure 4.3. Summary of the leaf movement temperature compensation for 18 *Arabidopsis* accessions.

Mean period estimates for individual leaves are plotted against their Rel. Amp. Error with black squares representing 17°C and white squares 27°C data. All seedlings were grown on MS agar under 12:12 L:D conditions at 22°C and after 10 days transferred to continuous light and either 17°C or 27°C where their circadian leaf movement rhythm was assessed. For each accession at each temperature n=30-40.

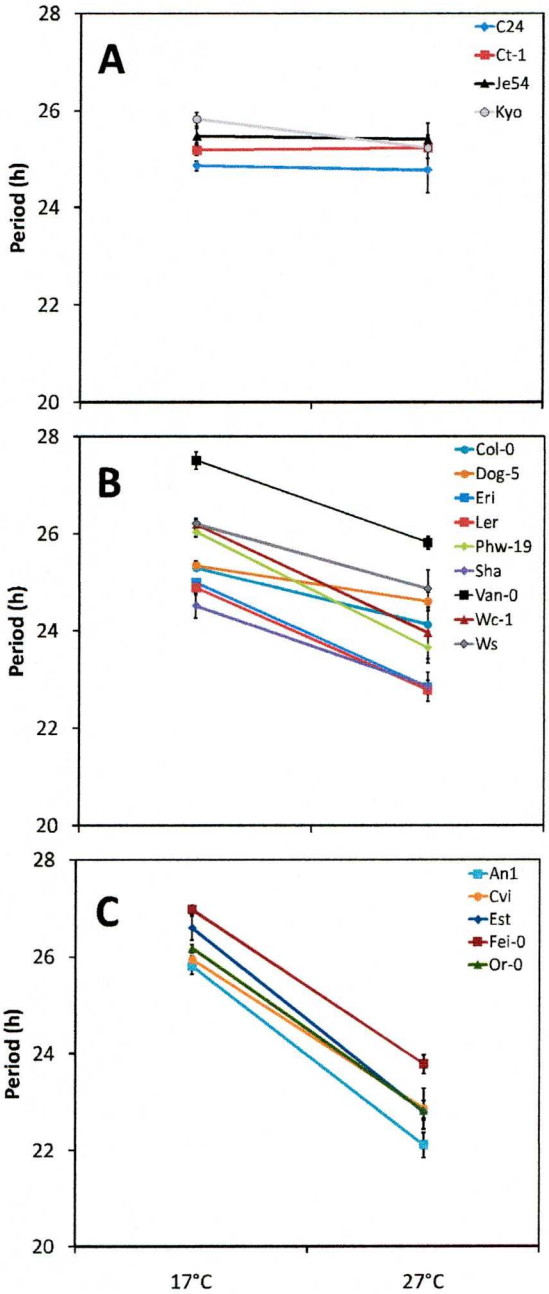
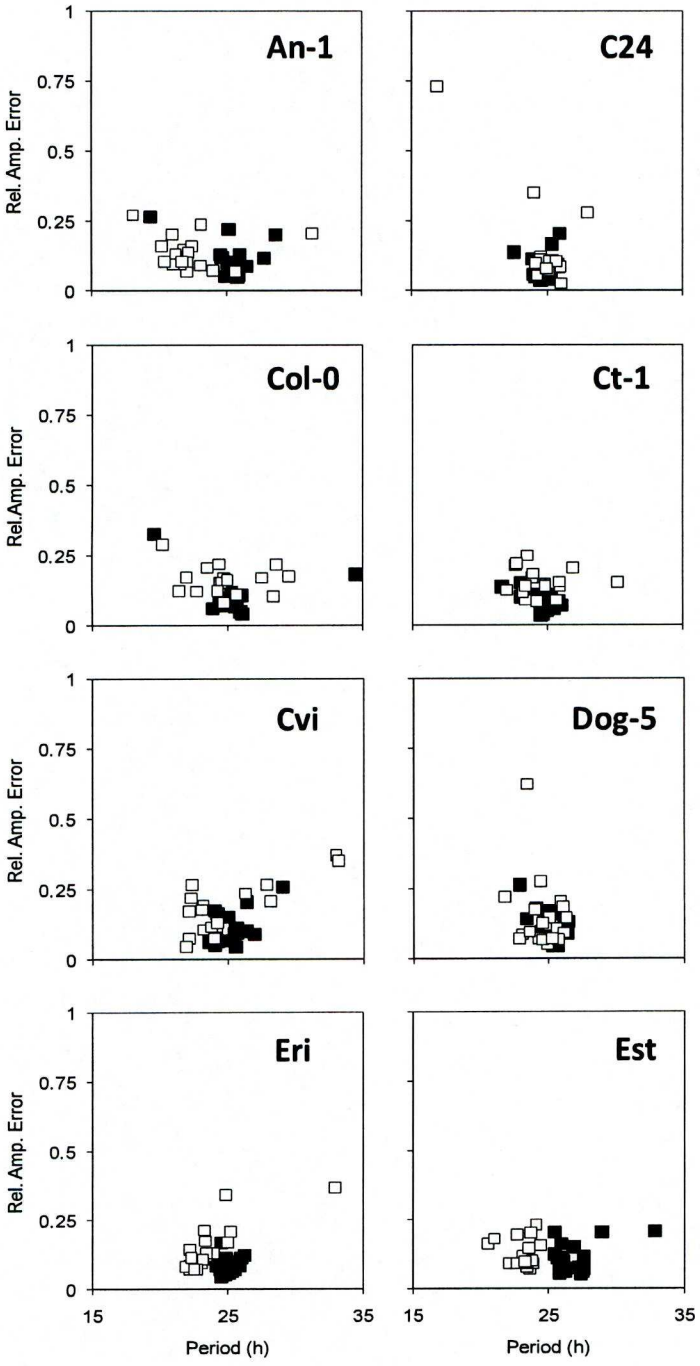
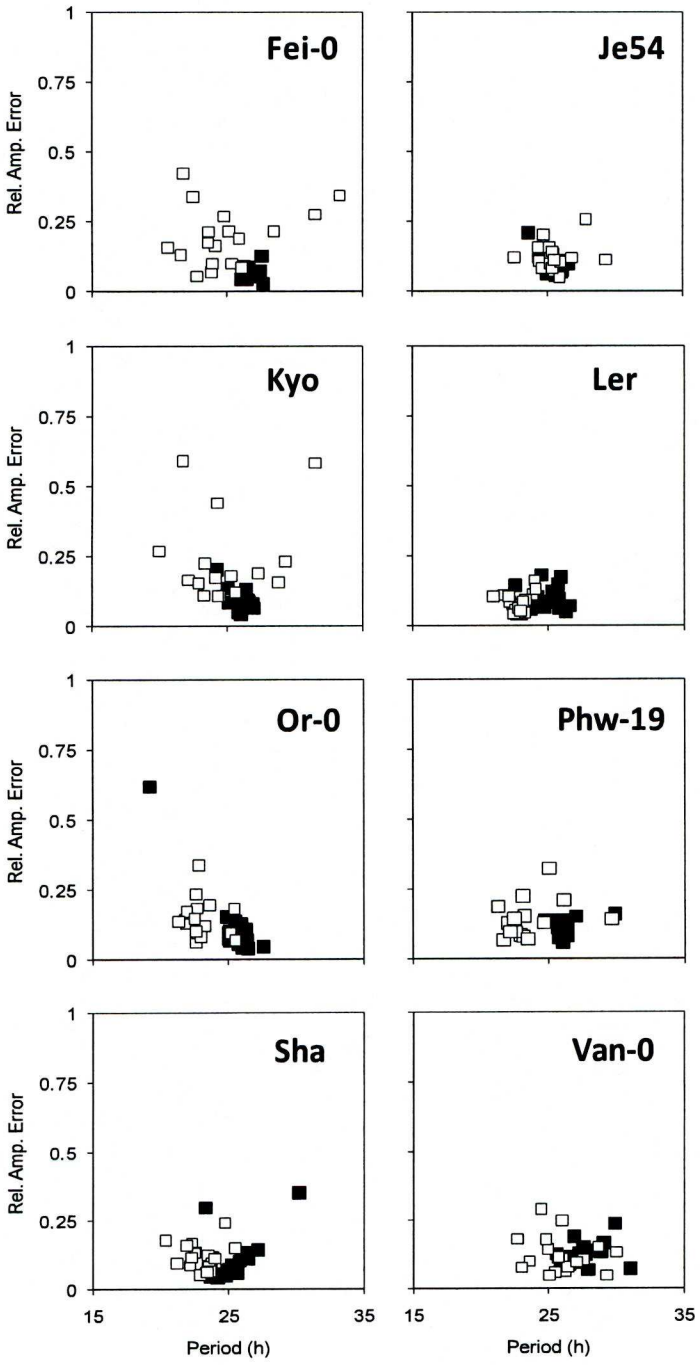


Figure 4.4. Circadian leaf movement period estimate (±SE) plotted against temperature.

Accessions are separated according to the temperature response: A-no significant difference in period between 17°C and 27°C; B-period shortening at 27°C, but decrease is less than 3 h; C- period shortening at 27°C with period decrease of more than 3 h. Period difference in accessions from B and C is statistical significant ($P<0.01$, except Col-0, where $P<0.02$). Plants were grown in 12:12 L:D for 10 days and then moved to 17°C or 27°C and continuous light where their free-running period was assessed.


An-1
C24
Col-0
Ct-1
Cvi
Dog-5
Eri
Est
Rel. Amp. Error
Rel.Amp. Error
0
0.25
0.5
0.75
1
15
25
35
Period (h)


Fei-0
Je54
Kyo
Ler
Or-0
Phw-19
Sha
Van-0
Rel. Amp. Error
1
0.75
0.5
0.25
0
15
25
35
Period (h)

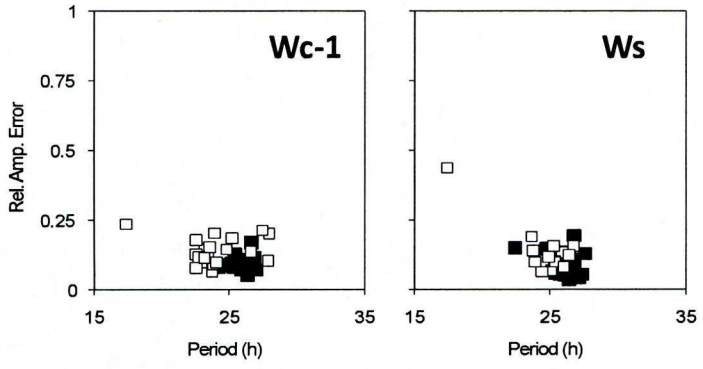


Figure 4.5. Effect of temperature on circadian leaf movement in different geographical accessions.

Plants were grown on MS agar under 12:12 L:D for 10 days before the transfer to 17°C or 27°C and continuous light, at which leaf movement rhythms were assessed. Scatter plots illustrate period estimates for each individual leaf plotted against its Rel. Amp. Error. Black squares, 17°C (n=20), open squares, 27°C (n=20). Accessions are presented in the alphabetic order.

Among the 9 accessions, plotted on Figure 4.4 B, the period decrease between 17°C and 27°C ranged from 0.7 h (in Dog-5) to 2.4 h (in Phw-19). Col-0 and Van-0 had an increase in period deviation from the mean, which is indicated by increased standard error (Table 4.1), leading to the wide spread of data points on the RAE plot (Figure 4.5). The remaining accessions exhibited a period decrease when monitored at 27°C in comparison to 17°C, though their rhythms were still precise, forming relatively tight clusters at each temperature. Accessions An1, Cvi, Est, Fei-0 and Or-0 were the most sensitive to temperature and displayed a more than 3 h period decrease when assessed at 27°C versus 17°C (Figure 4.4 C). In addition to the period shortening, Cvi and Fei-0 exhibited reduced precision of the clock.

Overall, natural variation in the circadian response to elevated temperature was observed in *Arabidopsis* accessions when assayed by the leaf movement technique. When combining all accessions together, a general trend towards period shortening with temperature increase was demonstrated, however, the degree of change in period and real amplitude error varied between individual accessions. While circadian periodicity of some accessions changed dramatically (more than 3 h), other accessions remained well temperature compensated and did not show significant changes in their circadian parameters. Furthermore, even though some accessions experienced period shortening, the precision of their circadian clocks remained unaffected. On the other hand, the clock precision in a few accessions decreased resulting in the reduced ability of individual leaves to move in unison with each other. In addition, no obvious correlation between period and robustness, and the thermo-sensitivity of accessions (determined earlier by J. Burke, pers. commun.) was established.

4.2.2.2.Relationship between leaf movement and geographical origin

Studies by Edwards et al. (2005) observed a correlation between longitude and circadian period at 22°C, but not at 12°C and 27°C. Here, as in Edwards et al. (2005), no correlation was present between circadian period and the latitude or longitude of accessions, at 27°C. The only parameters that exhibited significant correlation were longitude and period at 17°C (correlation coefficient of -0.546) (Figure 4.6). This is in agreement with the previously mentioned studies by Edwards *et al.* (2005), who speculated that such a correlation could be mediated by altitude. Altitude information was not available for many accessions used in the present study, therefore, a more in-depth relationship between circadian period and the geographical location of accessions could not be established.

4.2.3. Natural variation in temperature compensation using *LUC* bioluminescence assay

CCA1 and LHY are key components of the *Arabidopsis* circadian clock. Monitoring expression of the clock genes using a *LUC* reporter gene would provide evidence of the immediate temperature response associated with the circadian oscillator. It has been proposed that CCA1 and LHY contribute differently to the temperature compensation (Gould et al., 2006), therefore, monitoring expression of both genes across different accessions would provide a better understanding of the roles of CCA1 and LHY in temperature compensation and suggest if such a role differentiation is accession-specific.

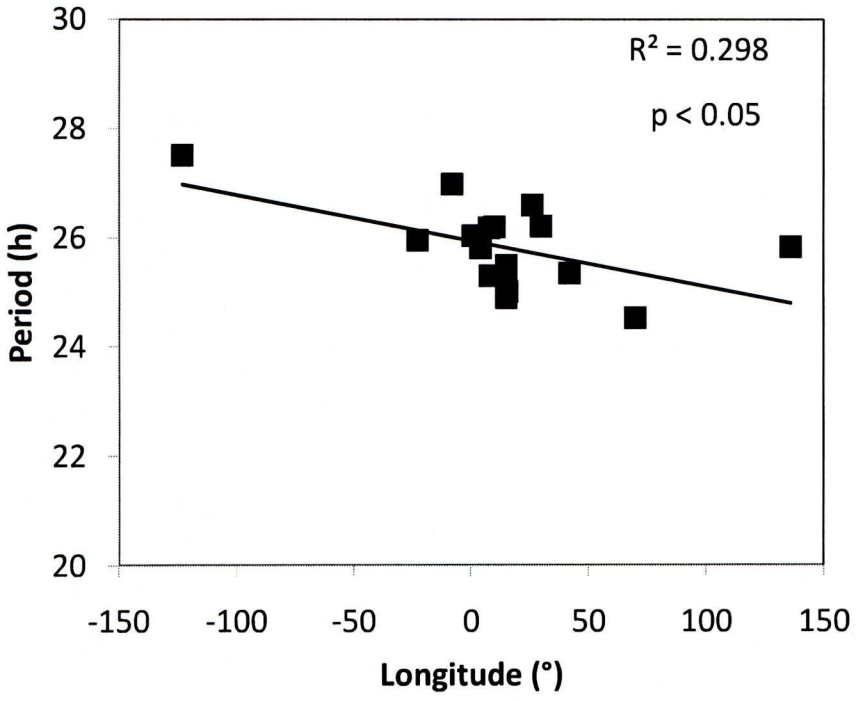


Figure 4.6. Correlation between leaf movement circadian period and accession longitude.

Each data point represents an average circadian period, calculated for individual accession at 17°C, plotted against the known latitude of the accession collection site. R^2 – correlation coefficient, p – probability.

All accessions used in the current study were separately transformed with *CCA1::LUC* and *LHY::LUC*, and screened on the appropriate antibiotic for successful transformants. Prior to the experiment, second generation (T_2) plant lines were selected based on the amount of light emitted from the transformed seedlings as well as the uniformity of oscillating rhythms between the lines. Uniformly oscillating and brightly glowing lines were favoured, with 4 lines representing each accession. Assayed seedlings were grown under 12:12 L:D conditions at 22°C. After 10 days they were transferred to continuous light at either 17°C or 27°C where their free-running periods were measured.

4.2.3.1. Variation in temperature response evaluated by CCA1::LUC.

Figure 4.7 is a frequency histogram of *CCA1* circadian period of accessions at 17°C and 27°C. At 17°C most of the accessions displayed 24 to 25 h rhythms. Surprisingly, no accessions kept the same period rhythmicity at 27°C as they did at 17°C, indicating that *CCA1* expression was temperature dependent (Table 4.2). All accessions were divided into 2 groups: 1) less than 4 h period change; and 2) more than 4 h period change (Figure 4.8). This was done primarily for graphical presentation convenience, as the period difference between the 2 temperatures ranged from 3.1 to 4.2 h for all accessions. In addition to the period alteration, all accessions experienced a decline in rhythm robustness, indicated by an increase in RAE values (Table 4.2). The overall RAE increase for all accessions combined was minor (Figure 4.9), nevertheless, some individual variation in the degree of RAE change associated with 27°C was observed. Figure 4.10 (left panel) illustrates individual RAE plots for *CCA1::LUC* expression at 17°C and 27°C.

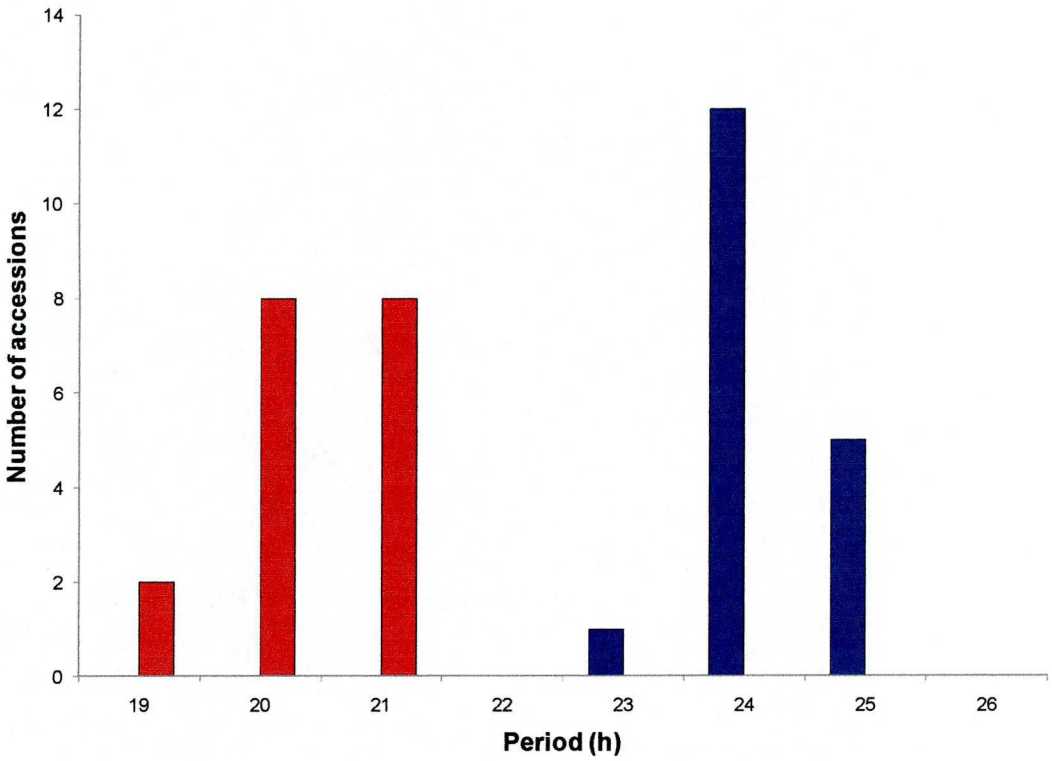


Figure 4.7. *CCA1* expression period distribution measured in 18 *Arabidopsis* accessions at 17°C (blue bars) and 27°C (red bars).

Table 4.2. *CCA1::LUC* biolumenescence period estimates (±SE) and RAE for 17°C and 27°C. SE – standard error.

	17°C			27°C		
Accession	Period (h)	SE	RAE	Period (h)	SE	RAE
An1	24.99	0.08	0.15	20.84	0.30	0.19
C24	25.13	0.08	0.16	21.56	0.44	0.27
Col-0	24.82	0.08	0.15	20.74	0.11	0.23
Ct-1	24.86	0.08	0.11	21.72	0.22	0.27
Cvi	24.50	0.11	0.16	20.64	0.06	0.33
Dog-5	25.12	0.04	0.16	21.74	0.09	0.21
Eri	24.03	0.03	0.10	19.94	0.12	0.30
Est	24.89	0.06	0.12	20.71	0.06	0.34
Fei-0	25.00	0.16	0.18	21.62	0.11	0.30
Je54	24.89	0.04	0.13	21.31	0.09	0.18
Kyo	24.55	0.09	0.22	20.48	0.18	0.28
Ler	24.33	0.04	0.13	20.00	0.07	0.18
Or-0	24.23	0.27	0.16	20.62	0.34	0.28
Phw-19	24.72	0.06	0.13	20.84	0.08	0.27
Sha	23.97	0.08	0.17	19.87	0.16	0.30
Van-0	25.01	0.24	0.14	21.45	0.16	0.27
Wc-1	25.16	0.07	0.15	21.79	0.08	0.17
Ws	24.77	0.03	0.15	21.61	0.06	0.21

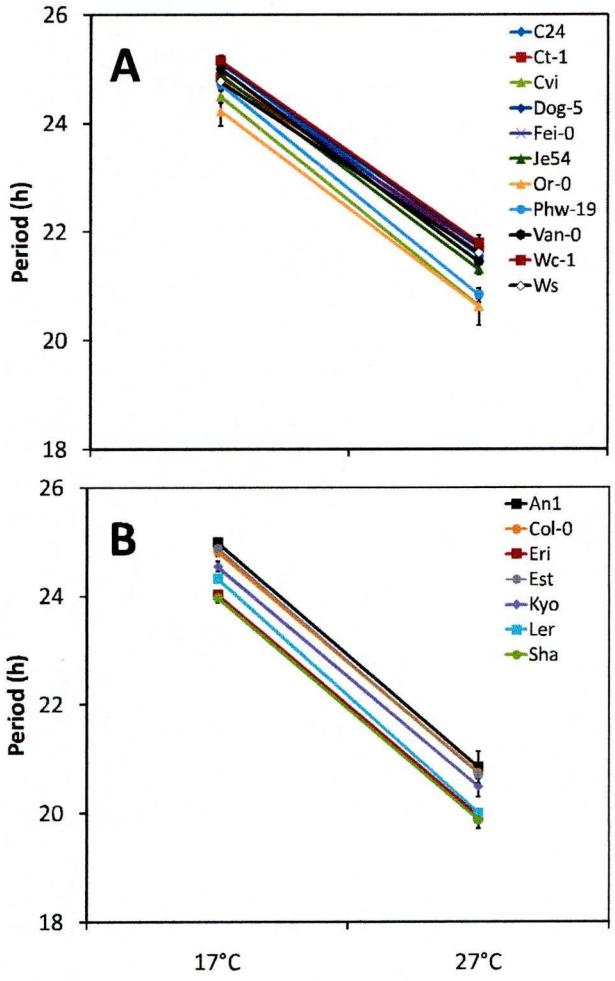


Figure 4.8. *CCA1::LUC* biolumenescence period estimate (±SE) plotted against temperature.

Accessions are separated according to the temperature response: A-period shortening from 17°C to 27°C is less than 4 h; B-period shortening at 27°C is more than 4 h. Plants were grown in 12:12 L:D for 10 days and then moved to 17°C or 27°C and continuous light where their free-running period was assessed.

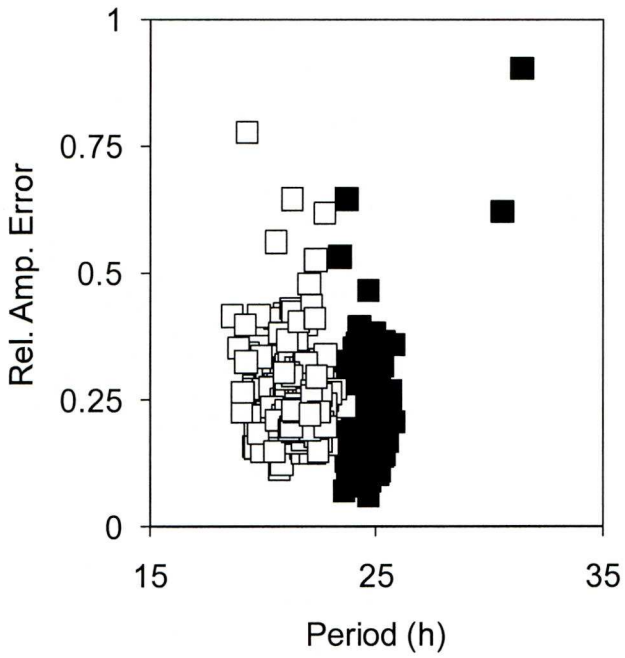
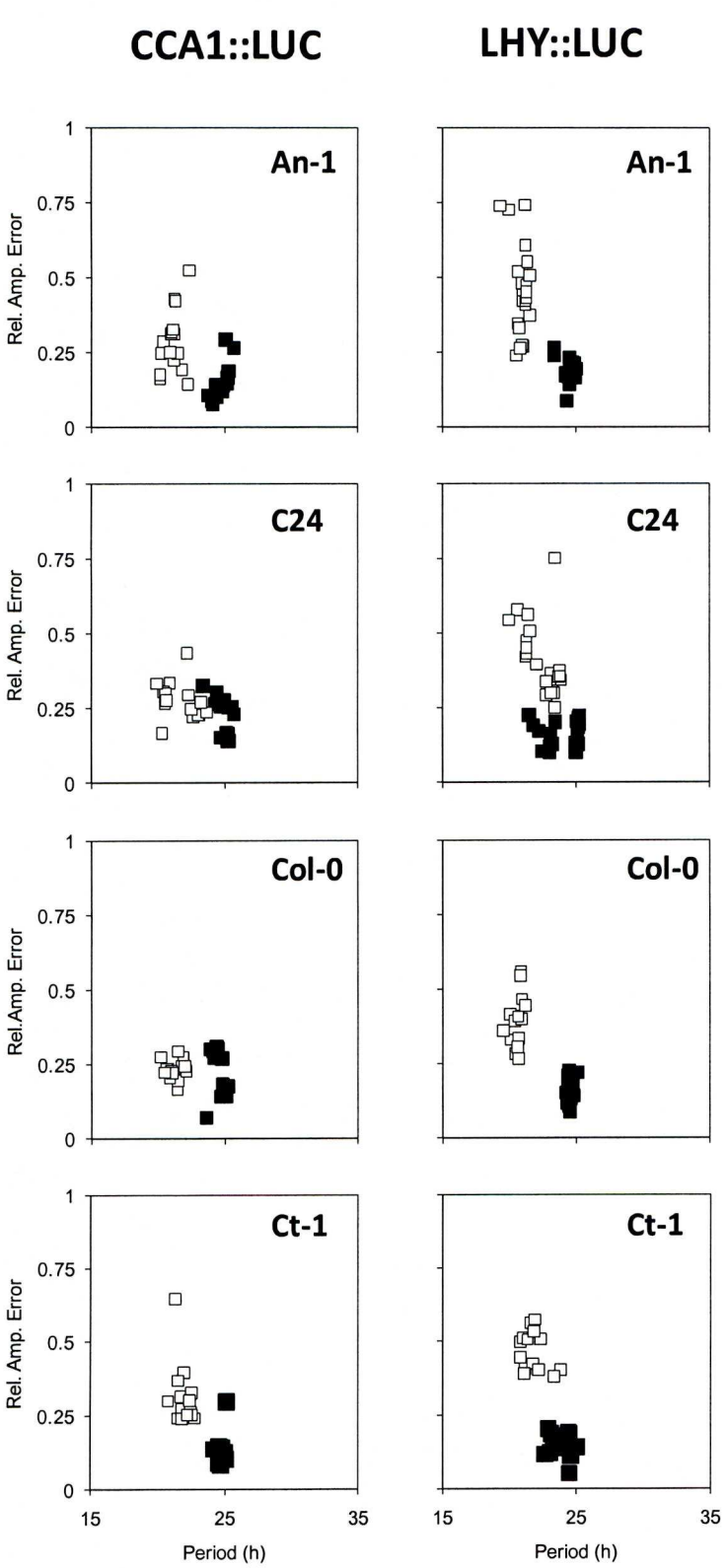
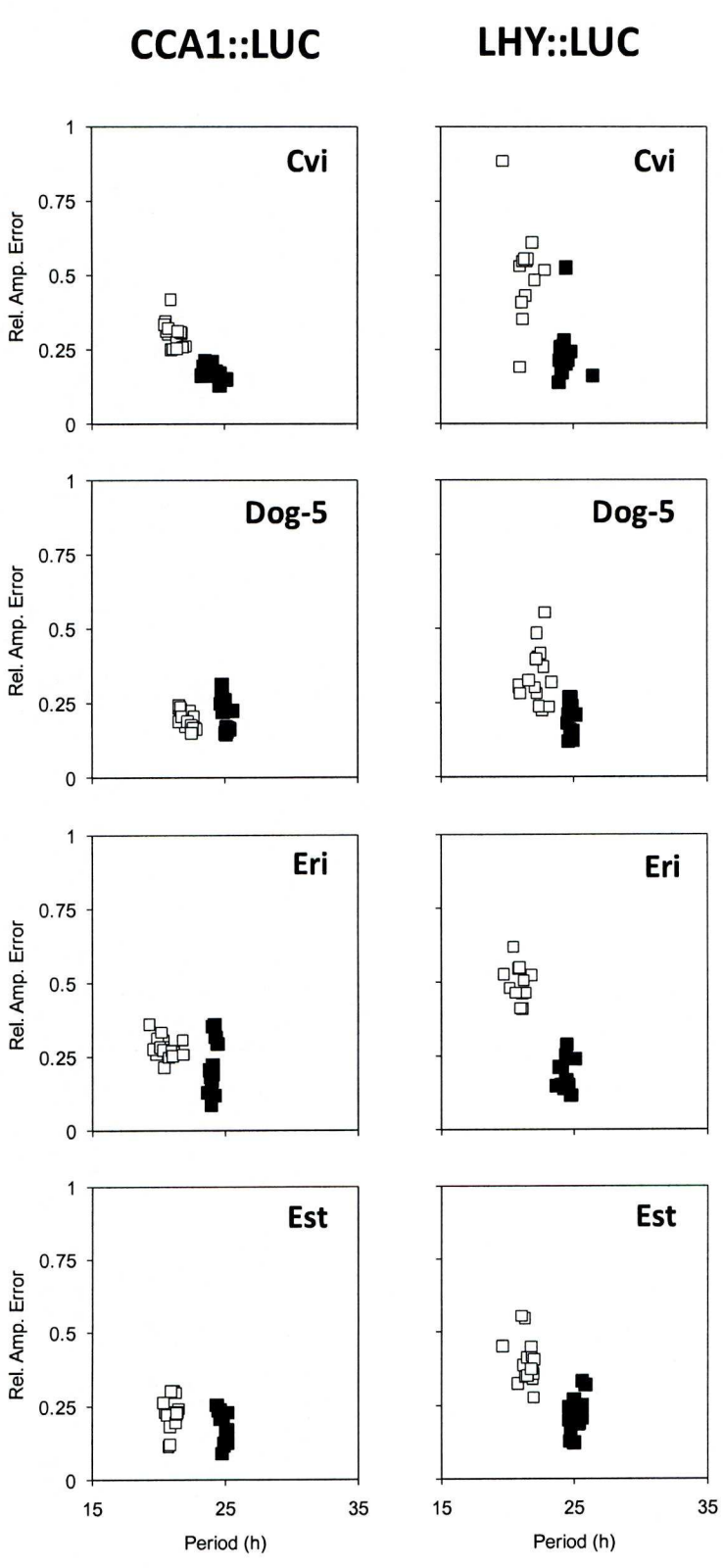
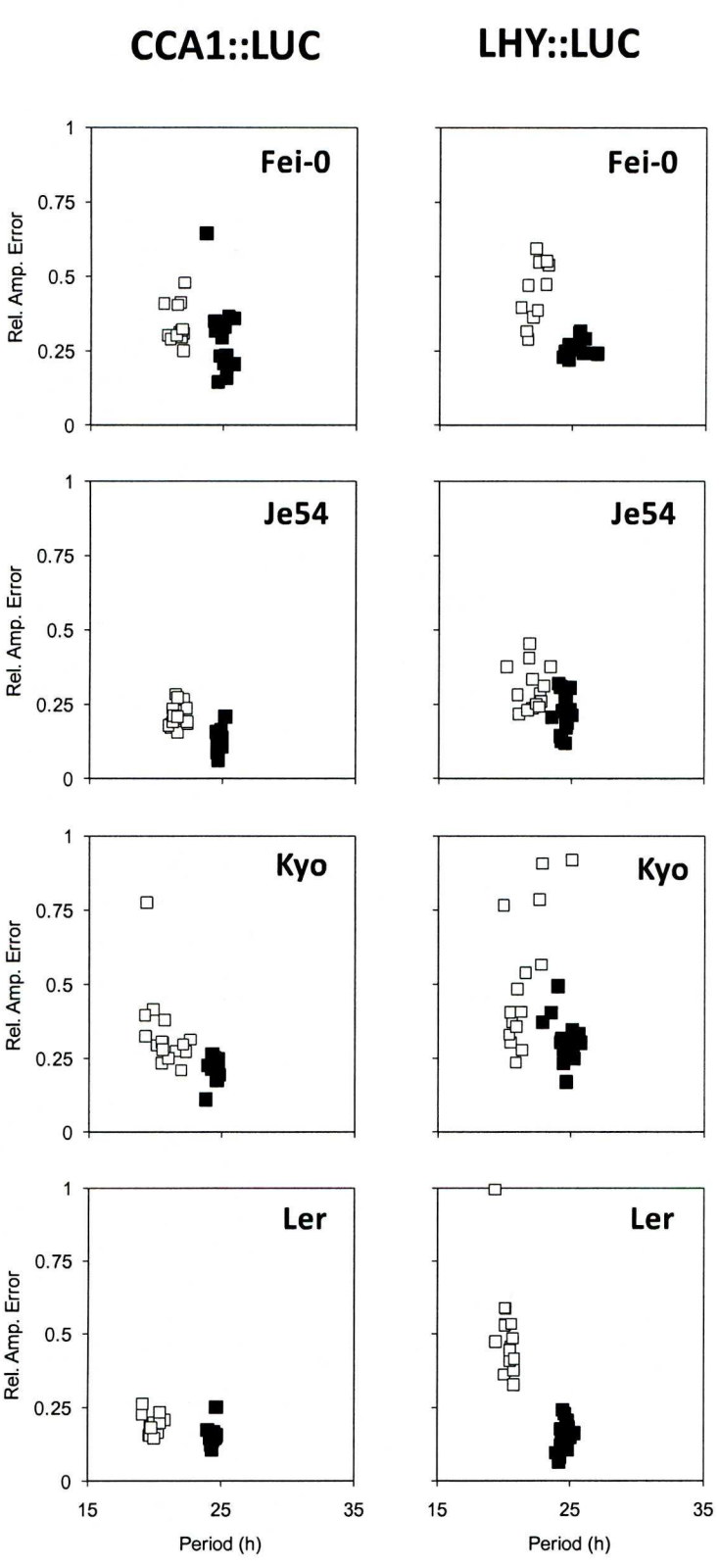


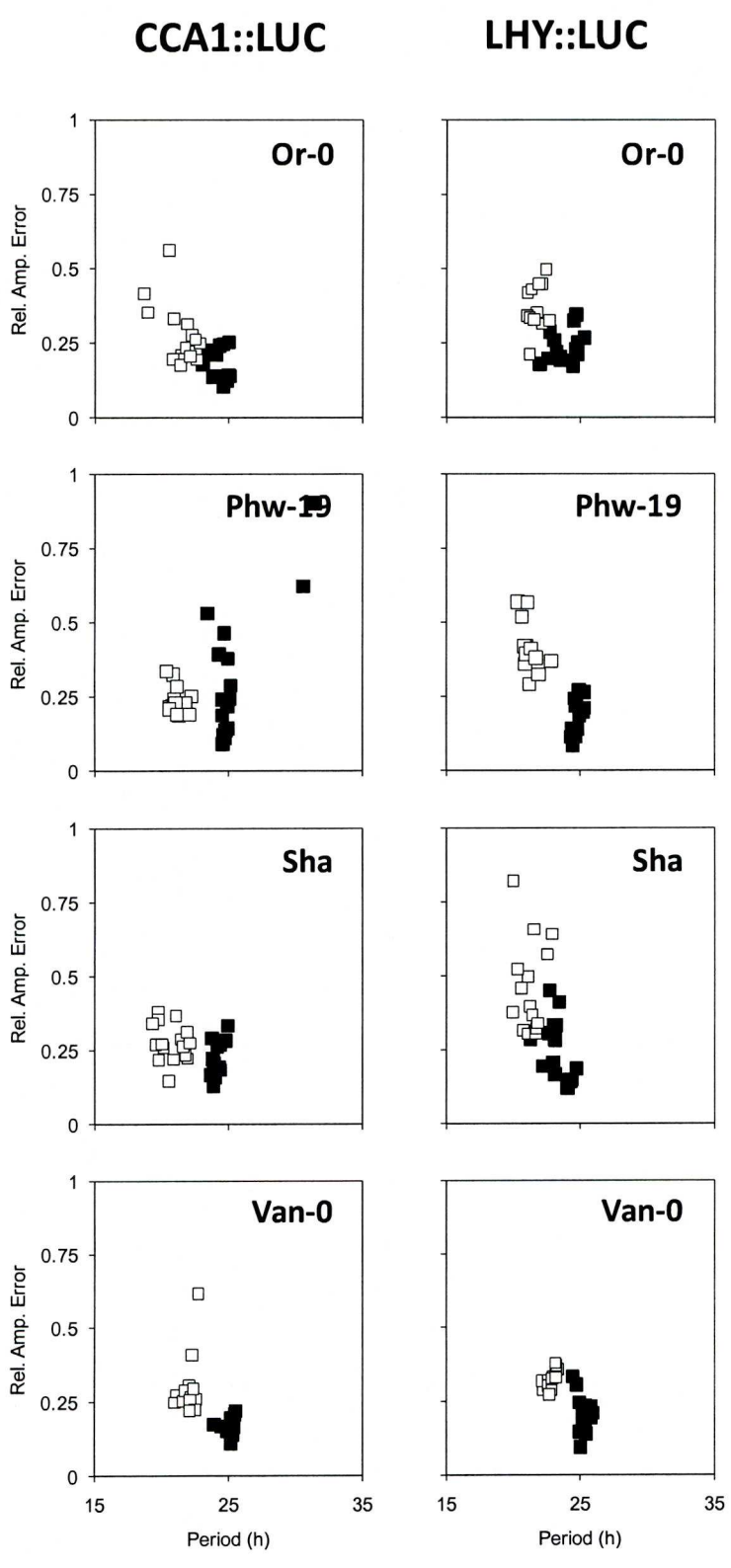
Figure 4.9. Summary of the temperature compensation in *CCA1* expression for 18 *Arabidopsis* accessions.

Mean period estimates for individual leaves are plotted against their Rel. Amp. Error (relative amplitude error) with black squares representing 17°C and white squares 27°C data. Groups of seedlings were grown under 12:12 L:D conditions at 22°C and after 10 days transferred to continuous light and either 17°C or 27°C where *CCA1::LUC* luminescence rhythms were assessed. For each accession at each temperature n=16.


CCA1::LUC
LHY::LUC
An-1
An-1
C24
C24
Col-0
Col-0
Ct-1
Ct-1
Rel. Amp. Error
Rel.Amp. Error
1
0.75
0.5
0.25
0
15
25
35
Period (h)


CCA1::LUC
LHY::LUC
Cvi
Cvi
Dog-5
Dog-5
Eri
Eri
Est
Est
Rel. Amp. Error
1
0.75
0.5
0.25
0
15
25
35
Period (h)


CCA1::LUC
LHY::LUC
Fei-0
Fei-0
Je54
Je54
Kyo
Kyo
Ler
Ler
Rel. Amp. Error
Rel. Amp. Error
Rel. Amp. Error
Rel. Amp. Error
0
0.25
0.5
0.75
1
15
25
35
Period (h)
Period (h)


CCA1::LUC
LHY::LUC
Or-0
Phw-19
Sha
Van-0
Rel. Amp. Error
1
0.75
0.5
0.25
0
15
25
35
Period (h)

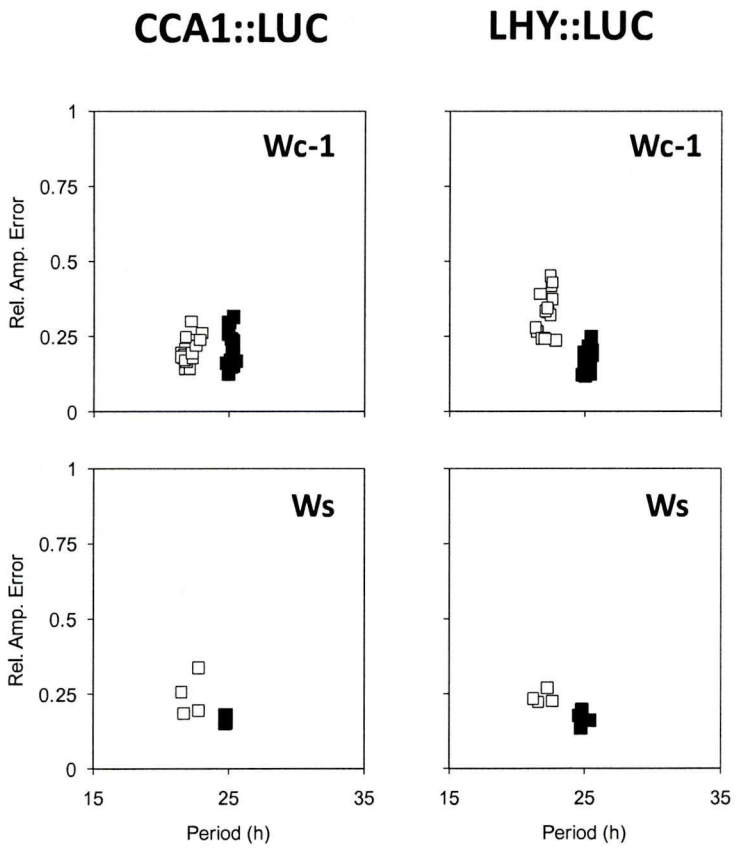


Figure 4.10. Effect of temperature on *CCA1* and *LHY* expression in different geographical accessions.

Plants were grown on MS agar under 12:12 L:D for 10 days before the transfer to 17°C or 27°C and continuous light, at which *CCA1* and *LHY* expression rhythms were assessed. Scatter plots illustrate period estimates for each individual group of seedlings plotted against its Rel. Amp. Error. Black squares, 17°C (n=16), open squares, 27°C (n=16). Plots on the left hand side represent *CCA1::LUC* expression, while plots on the right side are for *LHY::LUC* expression. Accessions are presented in the alphabetic order.

Visual analysis of the data clustering indicated that 9 accessions (Col-0, Cvi, Ct-1, Ler, Dog-5, Est, Je54, Van-0 and Wc-1) had robust rhythms over all temperature treatments, and subjection to 27°C caused period shortening only. On the other hand, temperature elevation had a different effect on An-1, C24, Fei-0, Kyo, Or-0, Sha and Ws, where it not only resulted in period shortening, but also caused a slight decline in rhythm robustness. Furthermore, the precision of the clock was disturbed in a subset of these (An-1, C24, Or-0 and Kyo), where at 27°C the data points were not clustered but spread out around the mean.

An attempt to correlate circadian *CCA1* expression with the geographical origin of accessions resulted in no statistically significant interaction between parameters at any temperatures.

4.2.3.2. Variation in temperature response evaluated by LHY::LUC.

Similarly to *CCA1::LUC* circadian period at 17°C, the majority of accessions displayed a 24 h period in *LHY::LUC* expression (Table 4.3). With the temperature increase, all accessions experienced circadian period shortening, however, the magnitude of this change was accession specific and ranged from 1.96 h in C24 and 4.07 h in Col-0. For Figure 4.11 accessions were divided into 2 groups: 1) less than 3 h period change; and 2) more than 3 h period change. In contrast to *CCA1::LUC*, none of the accessions displayed a rhythm of less than 20 h which is indicated by period frequency distribution histogram (Figure 4.12). In addition, only 3 accessions (Col-0, Kyo and Ler) had a period change of approximately 4 h at 27°C versus 17°C, while 7 accessions had a more than 4 h decrease for *CCA1::LUC* (Figure 4.11), suggesting that expression of *LHY* is better temperature compensated than expression of *CCA1*.

Table 4.3. *LHY::LUC* biolumenescence period estimates and RAE for 17°C and 27°C. SE – standard error.

	17°C			27°C		
Accession	Period (h)	SE	RAE	Period (h)	SE	RAE
An1	24.27	0.13	0.15	21.05	0.07	0.40
C24	23.90	0.50	0.13	21.94	0.49	0.50
Col-0	24.52	0.05	0.13	20.45	0.11	0.38
Ct-1	23.83	0.41	0.12	21.42	0.13	0.44
Cvi	24.26	0.25	0.19	21.73	0.33	0.57
Dog-5	24.80	0.05	0.15	21.43	0.26	0.29
Eri	24.20	0.09	0.18	20.90	0.27	0.49
Est	24.76	0.05	0.14	21.08	0.19	0.42
Fei-0	25.23	0.23	0.25	21.92	0.16	0.40
Je54	24.55	0.05	0.18	21.81	0.26	0.25
Kyo	24.49	0.29	0.27	20.54	0.11	0.37
Ler	24.21	0.05	0.10	20.25	0.20	0.47
Or-0	23.94	0.21	0.20	21.36	0.16	0.30
Phw-19	24.51	0.05	0.14	20.97	0.08	0.37
Sha	23.80	0.26	0.15	20.85	0.22	0.40
Van-0	25.11	0.08	0.18	22.56	0.11	0.31
Wc-1	25.00	0.05	0.15	21.80	0.12	0.27
Ws	24.69	0.04	0.15	21.39	0.12	0.23

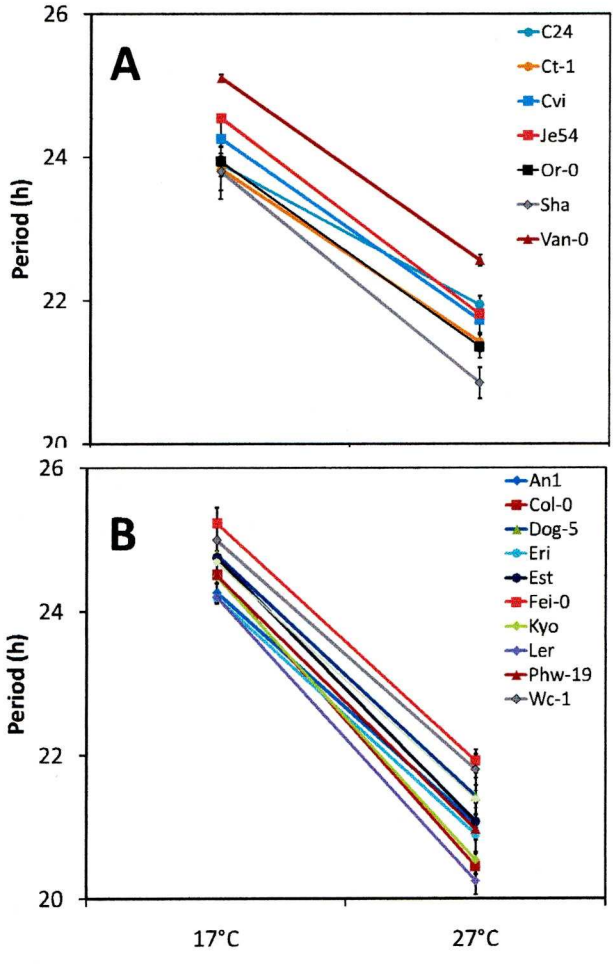


Figure 4.11. *LHY::LUC* biolumenescence period estimate (±SE) plotted against temperature.

Accessions are separated according to the temperature response: A-period shortening from 17°C to 27°C is less than 3 h; B-period shortening at 27°C is more than 3 h. Plants were grown in 12:12 L:D for 10 days and then moved to 17°C or 27°C and continuous light where their free-running period was assessed.

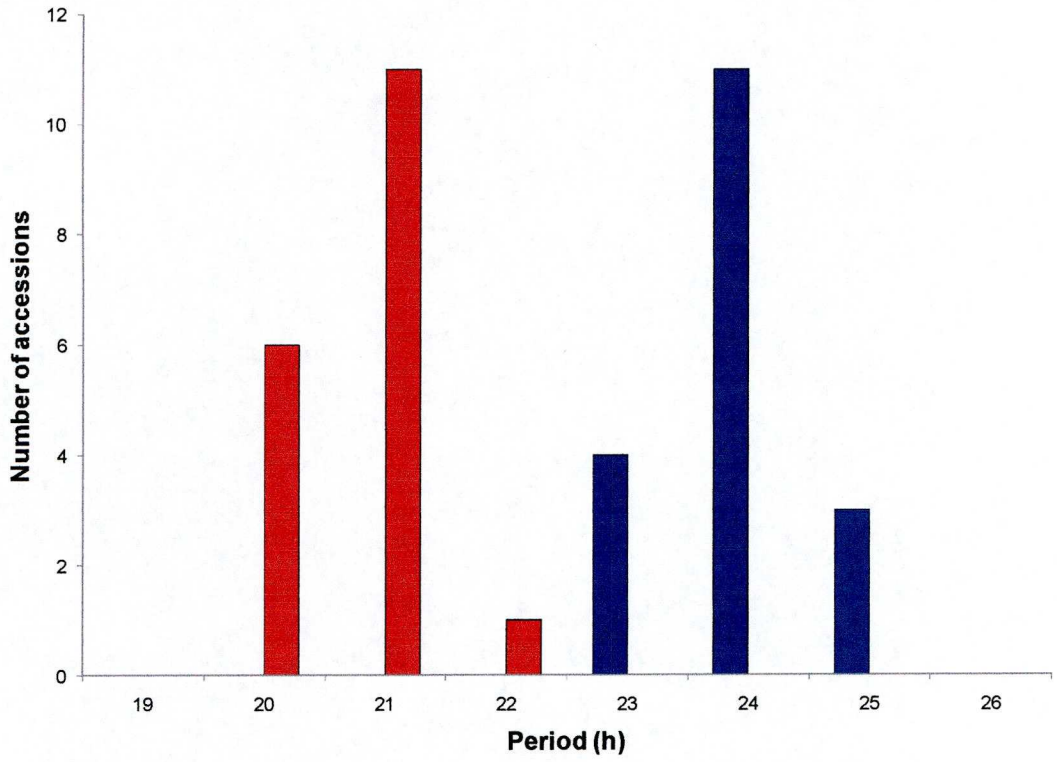


Figure 4.12. *LHY* expression period distribution measured in 18 *Arabidopsis* accessions at 17°C (blue bars) and 27°C (red bars).

Plotting all period data points against their RAE value at two temperatures showed a considerable decline in rhythm robustness with temperature increase (Figure 4.13). Furthermore, even though the degree of change in RAE values was accession specific, all of them displayed a several fold increase in this circadian parameter (Table 4.3). In general, 27°C treatment had a stronger effect on the rhythm robustness of the *LHY* expression, than it did on the period (Figure 4.13). For example, accessions C24, Ct-1 Or-0, Sha and Je54 displayed a more obvious increase in RAE than a decrease in the period, with 27°C period values overlapping with the 17°C (Figure 4.10, right panel). In the case of An-1, Col-0, Fei-0, Ler, Eri, Phw-19 and Wc-1 temperature had an effect on both period length and rhythm robustness (Figure 4.10).

Comparison of RAE or period change of *LHY* expression with the geographical origin of accessions revealed no significant correlation at any temperature. However, correlation was observed between longitude and the period, measured at 27°C (correlation coefficient = -0.647) (Figure 4.14). This relationship could be mediated by altitude which had been previously suggested by Edwards et al. (2005) and could be related to the proposed hypothesis that accessions from higher altitudes exhibit shorter period. Contrary to this proposal, no correlation between circadian period and longitude at 17°C was observed here.

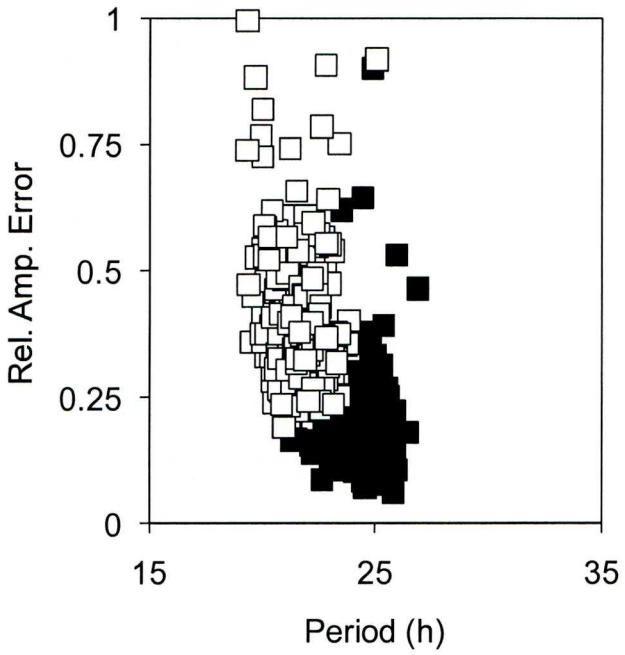


Figure 4.13. A summary of the temperature compensation in *LHY* expression for 18 *Arabidopsis* accessions.

Mean period estimates for individual leaves are plotted against their Rel. Amp. Error with black squares representing 17°C and white squares 27°C data. Groups of seedlings were grown under 12:12 L:D conditions at 22°C and after 10 days transferred to continuous light and either 17°C or 27°C where *CCA1::LUC* luminescence rhythms were assessed. For each accession at each temperature n=16.

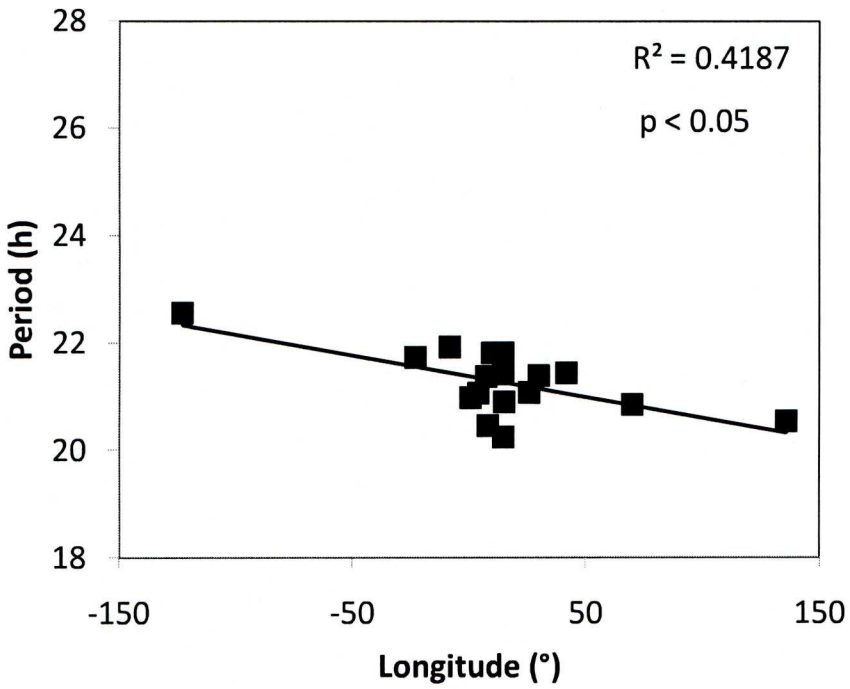


Figure 4.14. Correlation between *LHY* expression circadian period and accession longitude.

Each data point represents an average circadian period, calculated for individual accession at 27°C, plotted against the known latitude of the accession collection site. R^2 – correlation coefficient, p – probability.

4.2.3.3.Comparison of CCA1 and LHY expression

Examination of *CCA1* and *LHY* expression at 17°C to 27°C across accessions revealed that expression of the 2 circadian genes was temperature dependent. Furthermore, visual comparison of the *CCA1* and *LHY* data suggested that there was a difference in the effect of temperature increase between the two genes (Figure 4.9 and 4.13). The 27°C treatment mostly affected the period of the *CCA1* rhythms, shortening it by approximately 4 h across all accessions. Nevertheless, *CCA1* continued to oscillate robustly (Figure 4.9). In the case of *LHY*, its circadian period was also temperature sensitive, however often not to the same degree as for *CCA1* (Figure 4.13). On the other hand, 27°C had a much stronger effect on rhythm robustness, and in addition caused a decline in the precision of the clock in a number of accessions.

To determine whether the change in period and rhythm robustness was significantly affected by the circadian marker or temperature, and whether it was accession dependent, the general linear model using analysis of variance (ANOVA) was performed on the whole set of individual period and RAE values. Analysis of variation in response to the 27°C treatment, between the 18 accessions, revealed that both circadian period and RAE were significantly affected by temperature and this effect was accession specific (Table 4.4), indicating that accessions differed in their period and RAE response when subjected to the temperature treatment. Furthermore, all factors, such as temperature, accession, choice of marker and their combinations, which were used for ANOVA analysis, had a significant effect on rhythm robustness (Table 4.4). Figure 4.10, where RAE plots for 17°C and 27°C are presented separately for each marker and for each accession, shows the diversity of rhythmic response to temperature change.

Table 4.4. ANOVA of circadian period and RAE parameters in 18 *Arabidopsis* accessions.

d.f. – degree of freedom, SS – sum of squares, MS – mean square, F – F-statistics value, p – probability

Source	Period (h)					RAE				
	d.f.	SS	MS	F	p	d.f.	SS	MS	F	p
Accession	17	193.73	11.40	21.53	0.000	17	1.33	0.08	10.14	0.000
Marker	1	1.07	1.07	2.02	0.156	1	1.45	1.45	188.84	0.000
Temperature	1	2271.02	2271.02	4290.68	0.000	1	4.36	4.36	567.21	0.000
Accession*Marker	17	21.46	1.26	2.38	0.001	17	0.41	0.02	3.16	0.000
Accessoion*Temperature	17	85.06	5.00	9.45	0.000	17	0.64	0.00	4.86	0.000
Marker*Temperature	1	8.66	8.66	16.35	0.000	1	1.17	1.17	152.35	0.000
Accession*Marker*Temperature	17	22.75	1.34	2.53	0.001	17	0.51	0.03	3.94	0.000
Error	971	513.94	0.53							
Total	1042									

Notably, rhythm robustness was affected by the temperature in many accessions, with *LHY* often being more temperature-dependent than *CCA1* (e.g. C24, Cvi, Kyo).

No significant difference in circadian periods was found between *CCA1::LUC* and *LHY::LUC*, however the effect of accession*marker interaction was significant, indicating that the period of each marker was dependant on accession. In addition, the marker*temperature interaction was also significant, meaning that the temperature treatment influenced the markers differently. In order to identify accession dependant differences between *CCA1* and *LHY* expression periods, data averages for the two markers were plotted individually for each accession. Figure 4.15 illustrates that at 17°C, in the majority of cases, *CCA1::LUC* and *LHY::LUC* oscillate with similar periodicity. In accessions Cvi, Kyo, Sha and Van-0, *CCA1* and *LHY* oscillated with periods almost exactly matching one another, while in the rest of the accessions the difference between the two rhythms was usually not more than 20 min. Only in the accessions An1, C24 and Ct-1 did *LHY* oscillate slightly faster than *CCA1*, resulting in a 45min-1 h longer *CCA1* period.

Analysis of *CCA1::LUC* and *LHY::LUC* differences, between accessions at 27°C, revealed more cases where the periods of the 2 markers did not match (Figure 4.16). Je54 and Or-0 had a period difference of more than 30 min, while Cvi, Eri, Sha and Van-0 had more than 1 h difference. Interestingly, in all cases *CCA1* oscillated faster than *LHY*, therefore, resulting in a shorter period. It is worth mentioning that the variance of period was also affected by the temperature and generally had more impact on *LHY* than *CCA1* (Table 4.2, 4.3 and Figure 4.10).

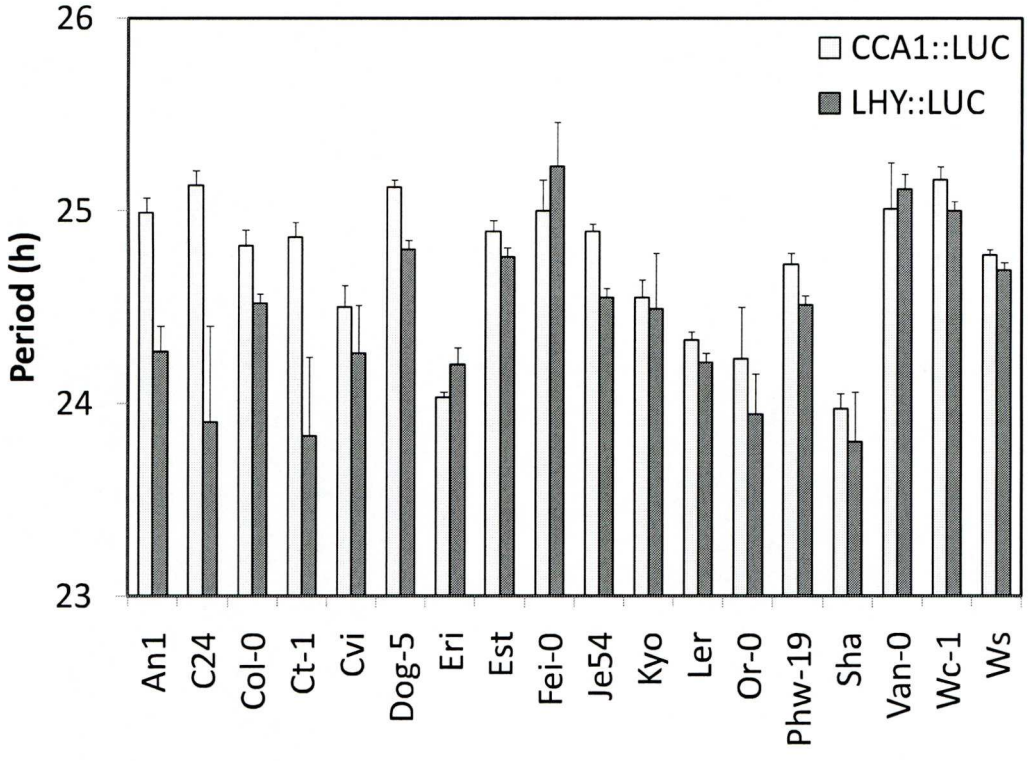


Figure 4.15. Comparison of *CCA1::LUC* and *LHY::LUC* average period estimates from 17°C between 18 *Arabidopsis* accessions.

Accessions, where the difference in period between *CCA1::LUC* and *LHY::LUC* is more than 30 min, are indicated with an arrow.

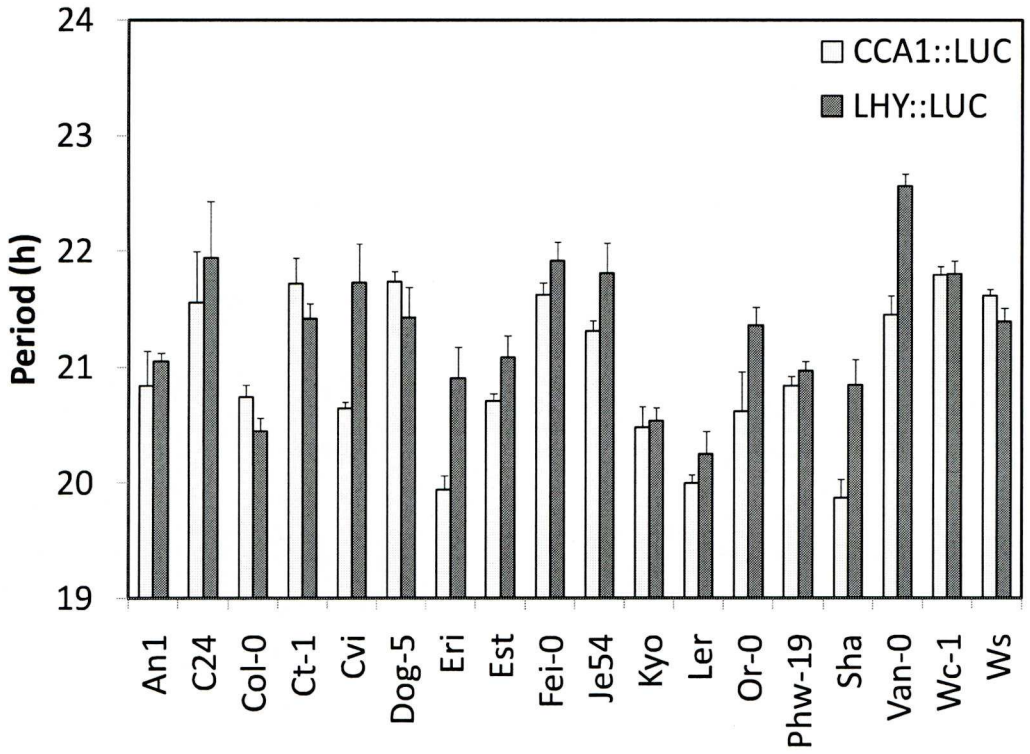


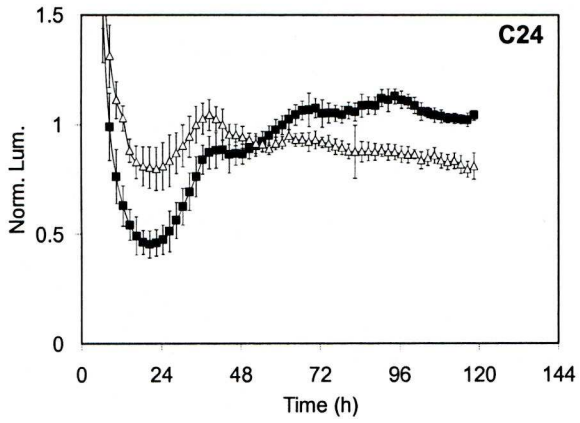
Figure 4.16. Comparison of *CCA1::LUC* and *LHY::LUC* average period estimates from 27°C between 18 *Arabidopsis* accessions.

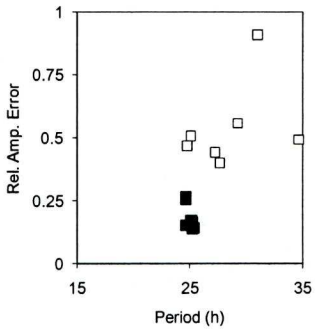
Accessions, where the difference in period between *CCA1::LUC* and *LHY::LUC* is more than 30 min, are indicated with an arrow.

Such an increase in the variation of the data could have had an influence on calculations of the average period estimates, consequently resulting in incorrect conclusions about *CCA1* and *LHY* period differences. However, possible functional differentiation of CCA1 and LHY, as well as uncoupling of circadian feedback loops, when subjected to high or low temperatures have been recently suggested (Gould et al., 2006 and A.Hall pers. commun.) and, therefore, the period divergence between *CCA1* and *LHY* in different accessions could be genuine.

4.2.3.4. Functional differentiation of CCA1 and LHY at 33°C

Expression of *CCA1* and *LHY* had similar circadian periods at 17°C (see section 4.2.3.3) which is in line with previous findings in the literature, and this has contributed to the notion that the two components have redundant roles. In contrast, at 27°C the circadian clock mechanism started to change, with *CCA1* and *LHY* free-running periods diverging from one another. However, the degree of the divergence between the two circadian genes was accession specific. To investigate if an even further loss of synchronisation between *CCA1* and *LHY* could be induced, and to determine the temperature range permissive for *CCA1* and *LHY* expression rhythmicity, all accessions were subjected to a 33°C treatment where expression of *CCA1::LUC* and *LHY::LUC* was monitored. Immediately after transfer to 33°C, expression of *LHY* became arrhythmic in all accessions (Figure 4.17). On the other hand, expression of *CCA1* while arrhythmic in some accessions, continued to oscillate in others (Figure 4.17 and Table 4.5). Such a split between *CCA1* and *LHY* rhythmicity suggests that 33°C triggers a change in the clock mechanism.


C24
Norm. Lum.
Time (h)
0
0.5
1
1.5
0
24
48
72
96
120
144


Rel. Amp. Error
Period (h)
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0.5
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1
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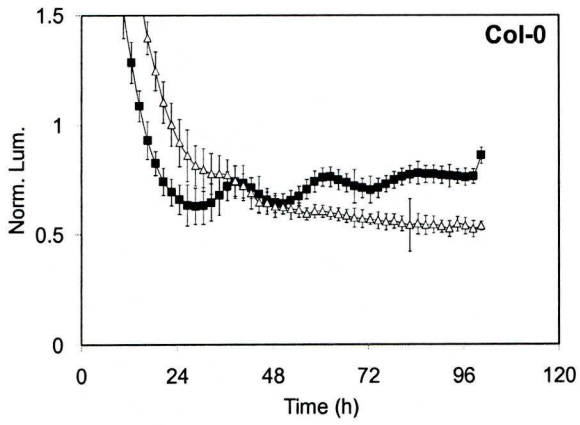
35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Norm. Lum.
Time (h)
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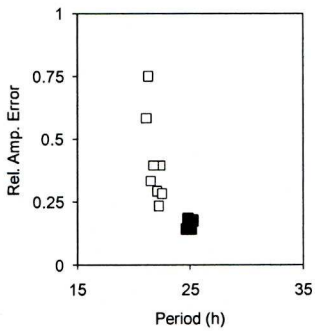
48
72
96
120


Rel. Amp. Error
Period (h)
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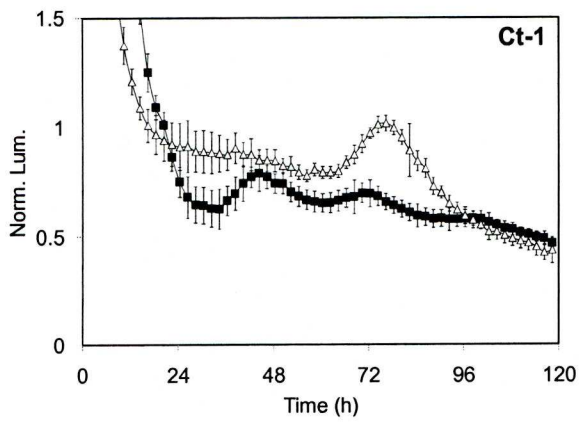
Ct-1
Norm. Lum.
Time (h)
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1.5
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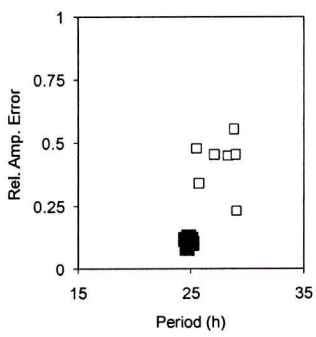
24
48
72
96
120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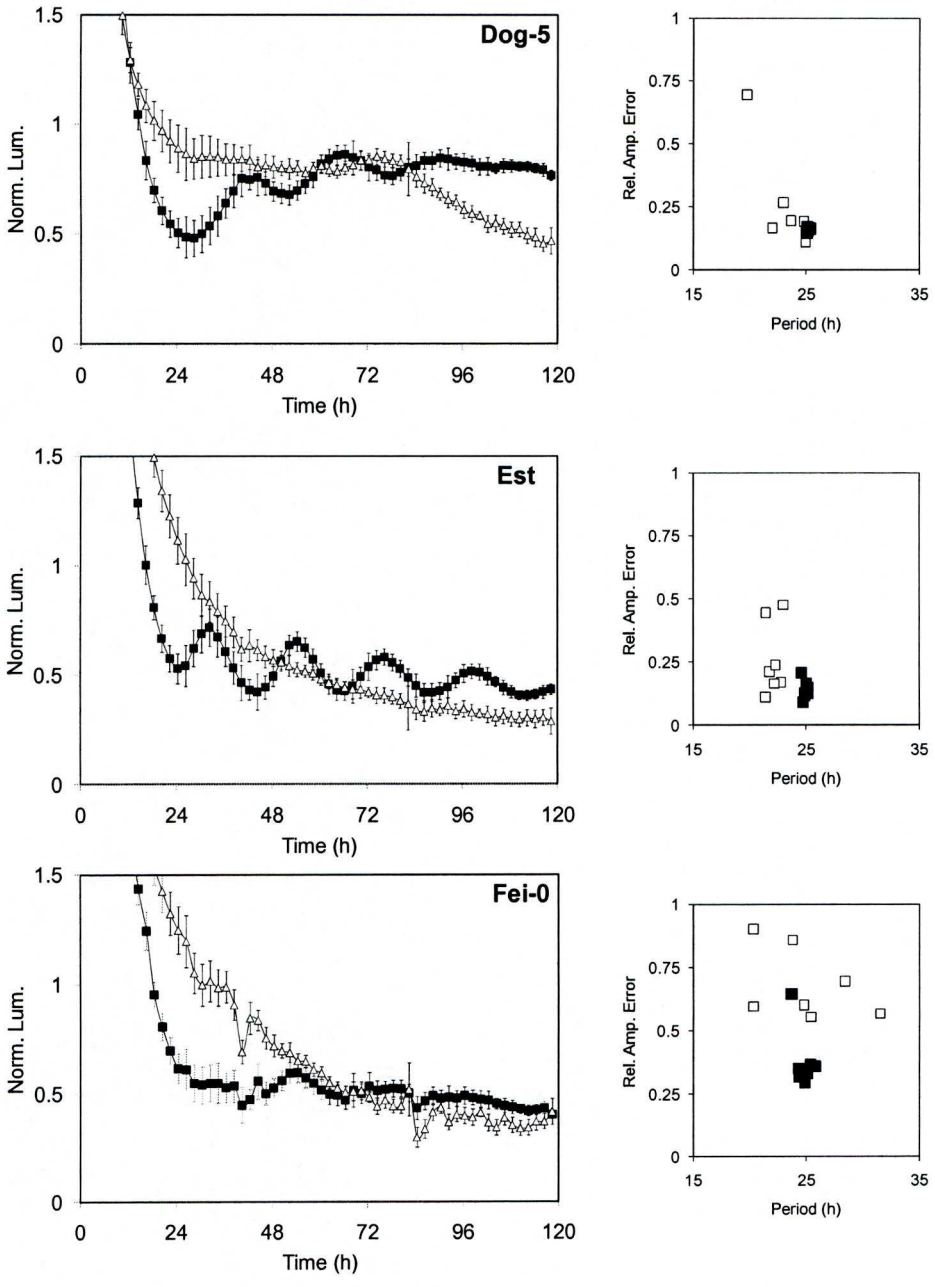
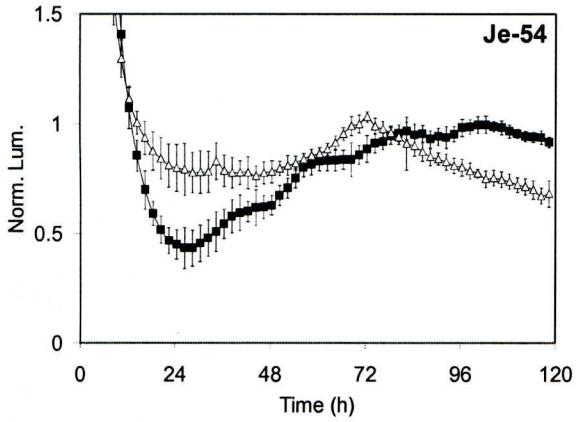

Dog-5
Norm. Lum.
Time (h)
Rel. Amp. Error
Period (h)
Est
Norm. Lum.
Time (h)
Rel. Amp. Error
Period (h)
Fei-0
Norm. Lum.
Time (h)
Rel. Amp. Error
Period (h)


Je-54
Norm. Lum.
Time (h)
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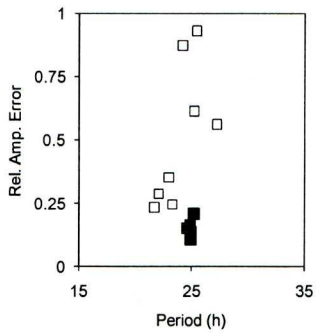
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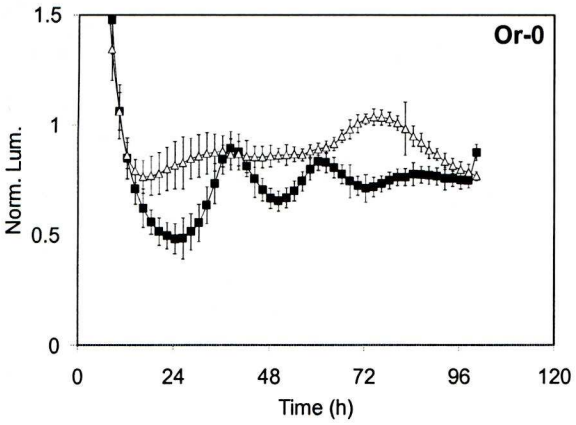
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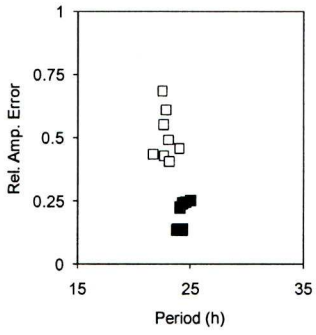
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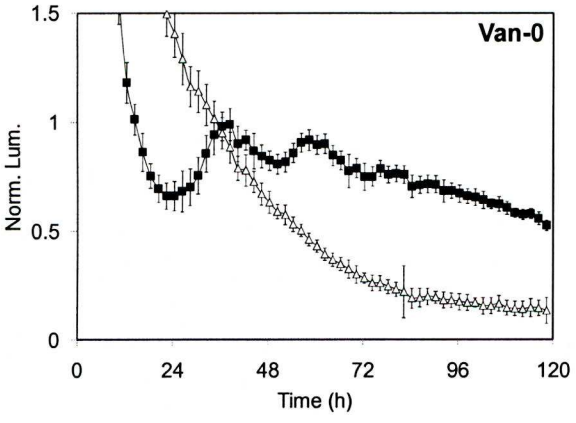
Van-0
Norm. Lum.
Time (h)
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1.5
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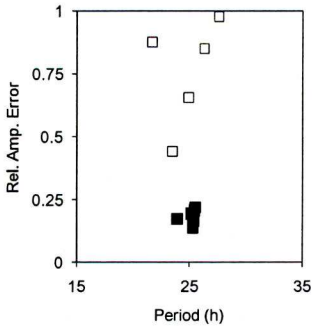
120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Period (h)
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0.75
1
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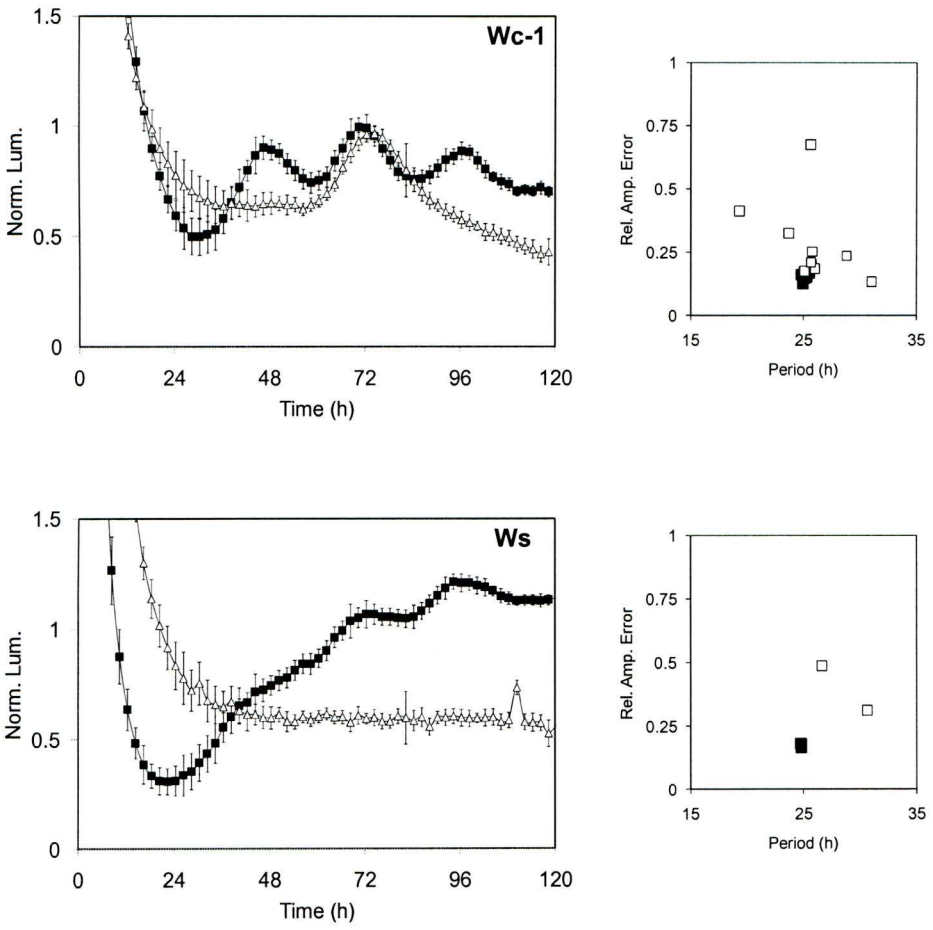


Figure 4.17. Effect of the 33°C treatment on *CCA1::LUC* and *LHY::LUC* expression.

Graphs on the left hand side represent normalized luminescence for *CCA1::LUC* (black squares) and *LHY::LUC* (open triangles). Seedlings were grown on MS agar under 12:12 L:D conditions at 22°C for 10 days before the transfer to 33°C and continuous light. Between 6 and 8 groups of seedlings were assayed per accession with the exception of Ws, where only 2 groups of seedlings were used. Plots on the right represent a comparison of *CCA1::LUC* expression at 17°C (black squares) and 33°C (empty squares). Data presented as period estimates for individual groups of seedlings plotted against their Rel. Amp. Error values.

Table 4.5. The average period estimate (±SE) and RAE of *CCA1* expression at 33°C. SE – standard error, RAE – relative amplitude error.

	33°C		
Accession	Period (h)	SE	RAE
An1	n/a	n/a	n/a
C24	27.02	0.65	0.48
Col-0	21.89	0.16	0.40
Ct-1	27.60	0.55	0.37
Cvi	n/a	n/a	n/a
Dog-5	23.78	0.54	0.19
Eri	n/a	n/a	n/a
Est	21.54	0.15	0.13
Fei-0	24.05	0.83	0.68
Je54	22.80	0.46	0.32
Kyo	n/a	n/a	n/a
Ler	n/a	n/a	n/a
Or-0	22.78	0.22	0.50
Phw-19	n/a	n/a	n/a
Sha	n/a	n/a	n/a
Van-0	23.83	0.65	0.61
Wc-1	25.49	0.54	0.21
Ws	28.12	1.39	0.42

It also appears that this clock mechanism is different across *Arabidopsis* accessions, where the period estimate for the 33°C *CCA1* rhythms varied considerably and resulted in short period and long period accessions (Table 4.5). Col-0, Est, Je54 and Or-0 exhibited short period rhythms ranging between 21.5 h to 22.8 h (Figure 4.17 and Table 4.5). The period variance of these accessions was relatively small implying that *CCA1* could maintain an accurate rhythm even at such elevated temperature. On the contrary, C24, Ct-1 and Ws exhibited a long period in *CCA1* expression. All 3 accessions together with accessions oscillating with a ~24 h rhythm produced high variance associated with the period (Figure 4.17 and Table 4.5). This result suggests that long-period accessions are more prone to the high temperature induced breakdown in the precision of the clock, whilst short period accessions are more temperature tolerant.

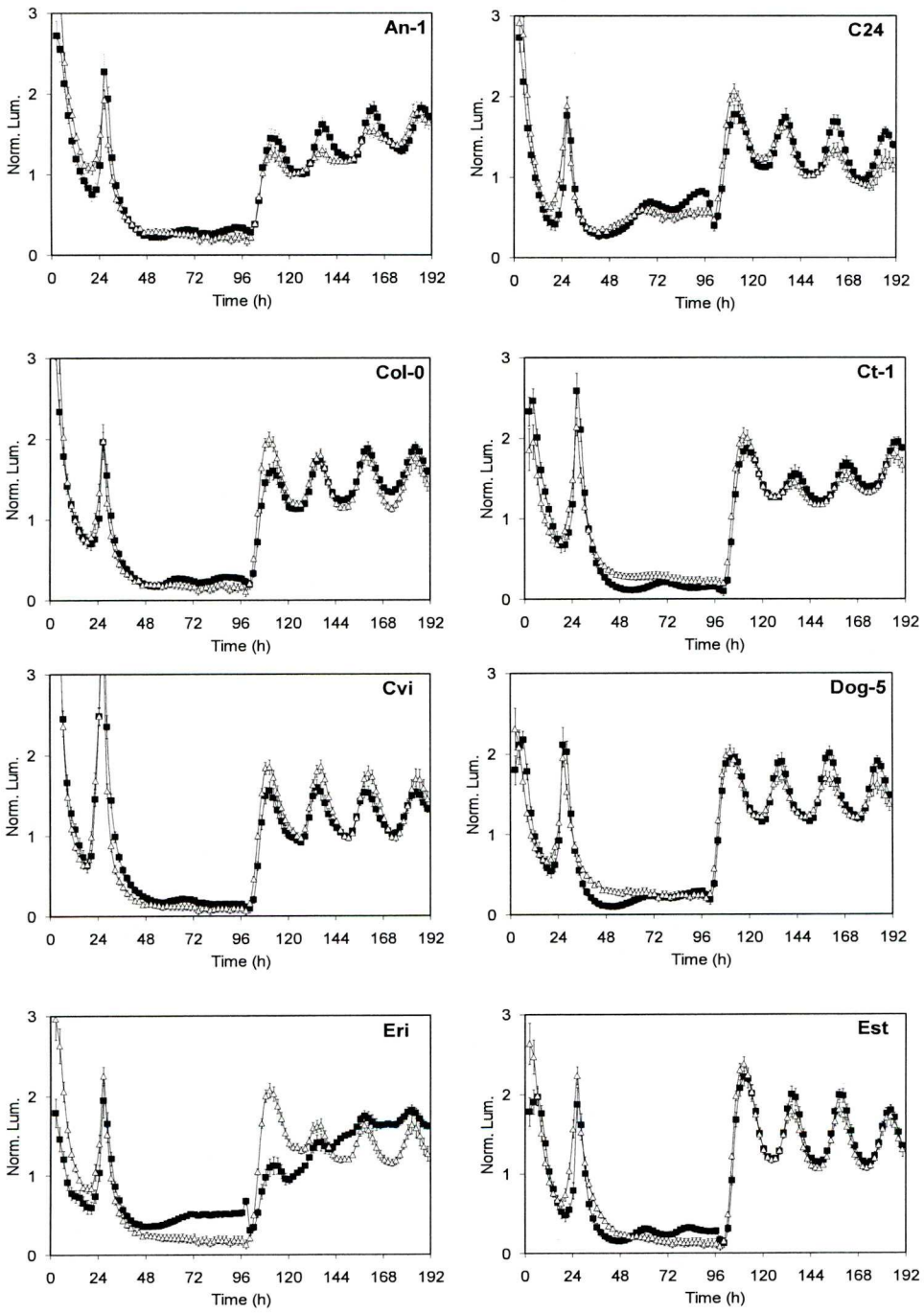
An increase in the variance of period as well as a decrease in the rhythm robustness at 33°C could in part be a result of elevated stress due to the increased growth temperature. At the end of the 33°C experiment (5 days), seedlings of all accessions appeared pale and damaged, indicating that 33°C was indeed highly stressful for the plants. Even after the transfer to the 22°C room many seedlings did not completely recover (personal observation). In any case, rhythmic oscillations of *CCA1* at 33°C in only a part of the examined accessions suggested that some accessions were more tolerant to the high temperature treatment, while others were temperature sensitive.

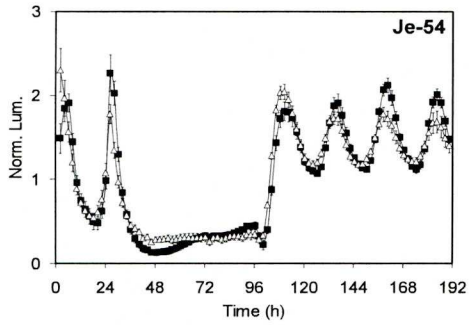
4.2.3.5. Analysis of CCA1 and LHY expression at 17°C and 33°C

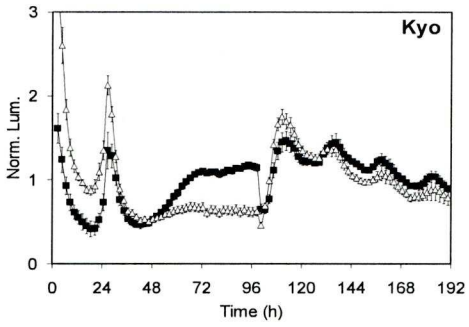
It is possible that exposure of temperature sensitive accessions to 33°C causes the breakdown of all physiological processes and/or plant death, therefore,

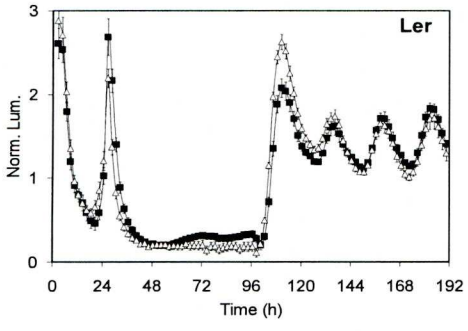
oscillations of expression of any genes would not be evident. On the other hand, 33°C treatment could have caused a temporary pause in some physiological processes, including circadian clock regulated oscillations, but these processes would resume upon transfer back to a non-stressful environment. To test this hypothesis, 10 day old *Arabidopsis* seedlings were first exposed to 17°C, then transferred to 33°C for 2 days, and moved back to 17°C for an additional 4 days. Expression of *CCA1::LUC* and *LHY::LUC* was monitored over the course of the entire experiment.

Figure 4.18 shows that on the first day (17°C) no difference between *CCA1* and *LHY* oscillations were noted across all accessions. However, when the temperature was raised to 33°C on the second day, the expression of both genes became suppressed with no detectable rhythm in *LHY* for all accessions and no rhythm in *CCA1* for half of the accessions. On the other hand, accessions described in the previous section as 33°C *CCA1* rhythmic, retained their rhythmicity. When the temperature in the growth chambers was switched back to 17°C, *CCA1* and *LHY* regained their expression patterns and continued to oscillate with an amplitude similar to the one from the first day of the experiment (Figure 4.18). The transition from 33°C to 17°C reset the clock to a dawn time point independent of whether a clock was maintained at 33°C or not. The resetting was also independent from the phase state the clock was at during the switch. This is especially obvious in Est (33°C free-running period=21.54 h), where during the temperature change from 33°C to 17°C, *CCA1* expression was at the beginning of the third cycle, while C24 (33°C free-running period=27.02 h) was only in the middle of the second cycle (Figure 4.18), confirming that various accessions oscillate at different periods when subjected to high temperatures.


An-1
C24
Col-0
Ct-1
Cvi
Dog-5
Eri
Est
Norm. Lum.
Time (h)
0
1
2
3
0 24 48 72 96 120 144 168 192


Je-54
Norm. Lum.
Time (h)
0
1
2
3
0 24 48 72 96 120 144 168 192


Kyo
Norm. Lum.
Time (h)
0
1
2
3
0 24 48 72 96 120 144 168 192


Ler
Norm. Lum.
Time (h)
0
1
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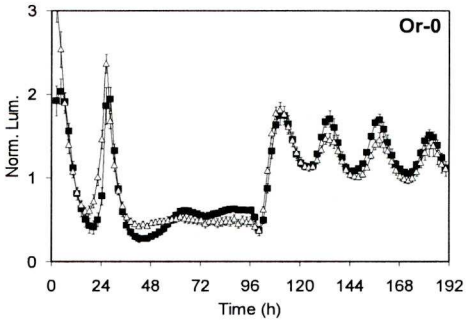
3
0 24 48 72 96 120 144 168 192


Or-0
Norm. Lum.
Time (h)
0
1
2
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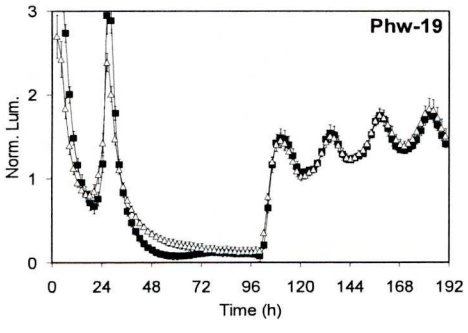
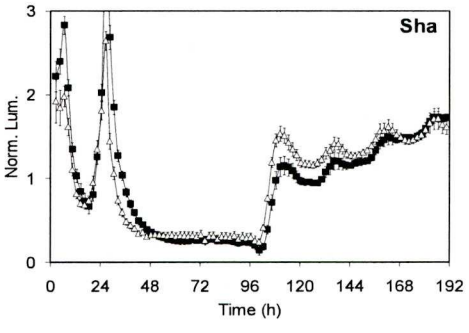
0 24 48 72 96 120 144 168 192


Phw-19
Norm. Lum.
Time (h)
0
1
2
3
0 24 48 72 96 120 144 168 192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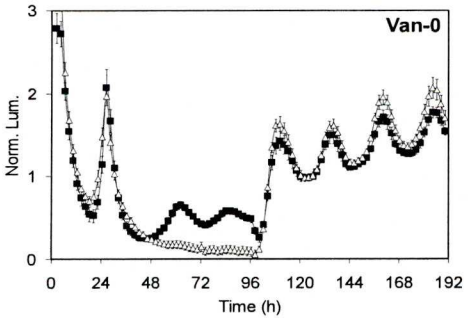
Van-0
Norm. Lum.
Time (h)
0
1
2
3
0 24 48 72 96 120 144 168 192

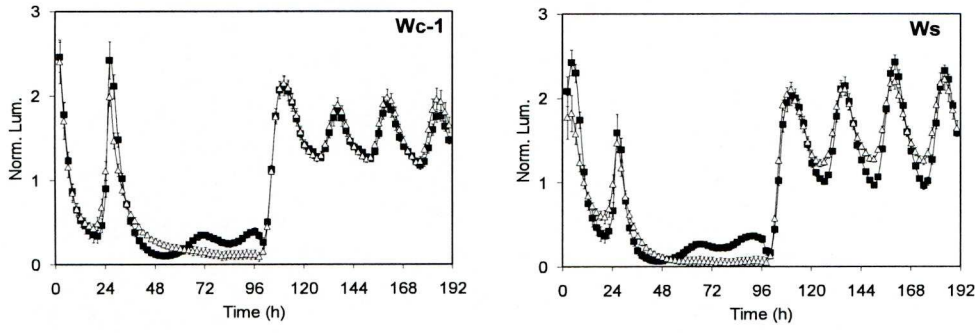

Figure 4.18. Effect of 33°C temperature on circadian clock performance measured by *CCA1::LUC* (black squares) and *LHY::LUC* (white triangles) biolumenescence.

Seedlings were grown on MS agar under 12:12 L:D conditions at 22°C for 10 days before the transfer to continuous light and 17°C. After 1 day the temperature was changed to 33°C for 3 days and then back to 17°C for additional 4 days. Graphs illustrate average normalized expression with standard error representing the range. N=8 groups of seedlings.

Furthermore, the 33°C to 17°C switch caused a brief, complete pause in *CCA1* expression, indicated by a drop in the *CCA1::LUC* luminescence. Such a drop was especially notable in accessions where *CCA1* was expressed at slightly higher levels at 33°C, e.g. C24, Eri and Kyo.

Overall, this experimental data suggest that 33°C does not irreversibly damage the circadian clock, but only temporary freezes or changes its function. It is possible that other genes/proteins or feedback loops take over the function of CCA1 and LHY and a variety of circadian clock outputs should be examined at 33°C to confirm this hypothesis. Leaf movement could not be used for this purpose due to the very fast growth of seedlings as well as high-temperature-induced flowering, which compromises the data analysis. In addition, the 33°C-17°C temperature switch experiment caused a complete re-start of *CCA1* and *LHY* expression in all accessions, resulting in the same phasing of oscillations and confirming the ubiquitous capability of temperature to entrain circadian rhythms.

4.2.4. Correlation between leaf movement, *CCA1* and *LHY* expression

To determine whether any relationship exists between the circadian clock outputs measured at 17°C and 27°C, Pearson correlations were calculated between all circadian period estimates. At 17°C a weak correlation between leaf movement and *LHY* expression was detected (correlation coefficient=0.55) (Table 4.6). However, no significant correlation was found between leaf movement and the *CCA1* expression period. In contrast, the circadian period of *CCA1* strongly correlated with *LHY*, confirming close interaction between the two circadian genes.

Table 4.6. Pearson correlation matrix for circadian clock output periods at 17°C and 27°C.

Cells in red indicate no correlation, *- indicates levels of statistically significant correlation where *- $p<0.05$, **- $p<0.01$, ***-$p<0.001$.

	Leaf Movement 17C	CCA1::LUC 17C	LHY::LUC 17C	Leaf Movement 27C	CCA1::LUC 27C	LHY::LUC 27C
Leaf Movement 17C		0.415	0.550*	0.183	0.406	0.603**
CCA1::LUC 17C	0.415		0.742***	0.488*	0.866***	0.533*
LHY::LUC 17C	0.550*	0.742***		0.300	0.477	0.514*
Leaf Movement 27C	0.183	0.488*	0.300		0.634**	0.430
CCA1::LUC 27C	0.406	0.866***	0.477*	0.634**		0.693***
LHY::LUC 27C	0.603**	0.533*	0.514*	0.430	0.693***	

This interaction was only slightly weakened by the 27°C treatment. Expression of *CCA1* at 17°C strongly correlated with *CCA1* expression at 27°C (Figure 4.19 A), whilst the relationship was much weaker between 17°C and 27°C *LHY* expression, supporting earlier observations that the period change between *CCA1* expression at 17°C versus 27°C was more uniform across all accessions compared to *LHY*, where it was more accession specific. Interestingly, expression of *CCA1* at 17°C correlated with all circadian outputs measured at 27°C (Table 4.6), with various degrees of significance, indicating that *CCA1* would probably be a better indicator of circadian clock functioning. However, according to the regression plots (Figure 4.19), less than 30% of *LHY* expression and leaf movement at 27°C could be explained by 17°C *CCA1* data using the linear regression model. Keeping that in mind, together with the existence of the between accession variation in *CCA1::LUC* at 17°C, it appears that prediction of temperature induced changes in the circadian clock based on *CCA1* expression (at 17°C) only, would be highly capricious. Moreover, the change in leaf movement or expression of *LHY* at 27°C examined here was very accession specific as probably are any other circadian mediated outputs not investigated here.

4.2.5. Variation in high temperature growth performance in *Arabidopsis* accessions

It has been recently shown that at 22°C an endogenous circadian clock matching the light dark environment is beneficial for plants and positively affects their performance (Dodd et al., 2005). To determine the effect of temperature and circadian clock on plant growth performance at 27°C, as well as to investigate if any link exists between temperature dependent circadian clock variation and *Arabidopsis* growth, fresh and dry weight of accessions grown at 17°C and 27°C were assessed.

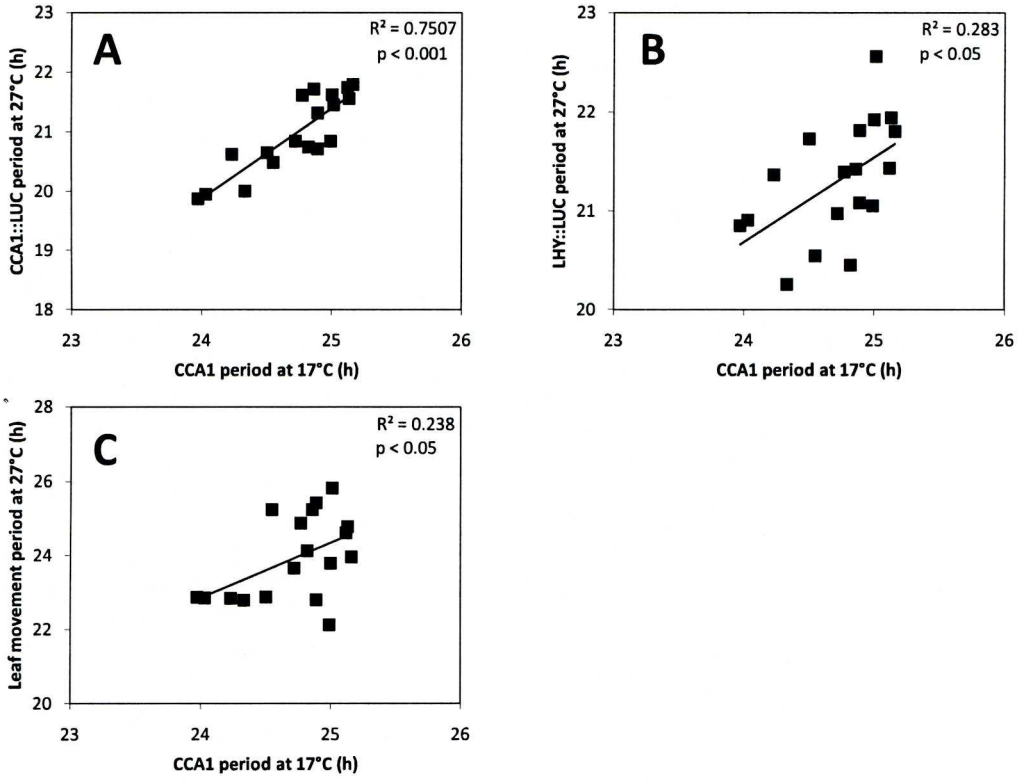

Figure 4.19. Correlation between circadian period of *CCA1* expression at 17°C and circadian periods of *CCA1* (A), *LHY* (B) and leaf movement (C) at 27°C.

Total weight was chosen for the assay as it is relatively easy to measure and is a good indicator of plant growth performance in response to stress.

All accessions were stratified at 4°C and then grown in soil under 12:12 L:D at 22°C for 12 days. Seedlings were then transplanted into individual 20-cell trays and, after an additional 2 days at 22°C, were moved to growth cabinets with temperatures set for 17°C or 27°C. All accessions were harvested after 14 days in the temperature treatment, prior to plant bolting. The growth performance experiment was repeated twice. Both times fresh weight and dry weight were determined for each seedling from every accession. This was done to check whether temperature directly affects plant growth or plant water uptake. Fresh weight values strongly correlated with dry weight both at 17°C (correlation coefficient=0.741) and 27°C (correlation coefficient= 0.938) (Figure 4.20) suggesting that dry weight measurements were sufficient for this experiment.

High temperature promoted growth in all accessions (Figure 4.21). It also affected the phenotypical appearance of plants by causing elongation of petioles and, in some cases, leaves (Figure 4.22). Nevertheless, considerable variation among accessions was observed in growth response to high temperature. Variation between the 2 experiments conducted a month apart from one another also occurred and will be discussed further on. At 17°C plant size variation between accessions was observed, therefore, weight gain, as opposed to raw data, was used to represent the weight increase associated with the 27°C treatment. Weight gain was calculated by subtracting the mean weight at 17°C from the mean weight at 27°C, and fresh and dry weight gain was calculated separately for each accession. Relative weight gain was not used as it revealed the same trend (data not shown).

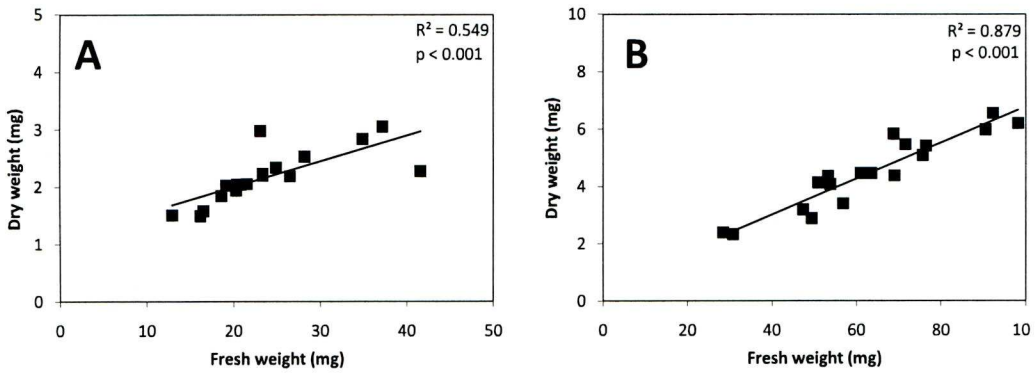

Figure 4.20. Correlation between the fresh and dry weight of *Arabidopsis* accessions.

Seedlings were grown in soil at 22°C under 12:12 L:D conditions. After 12 days, seedlings were individually transplanted into 20-cell trays and transferred into growth chambers with temperature set to 17°C (A) and 27°C (B) and 12:12 L:D. All plants were harvested and weighted after 14 days in the appropriate temperature treatment. To determine dry weight, same seedlings were dried in the oven for 48 h.

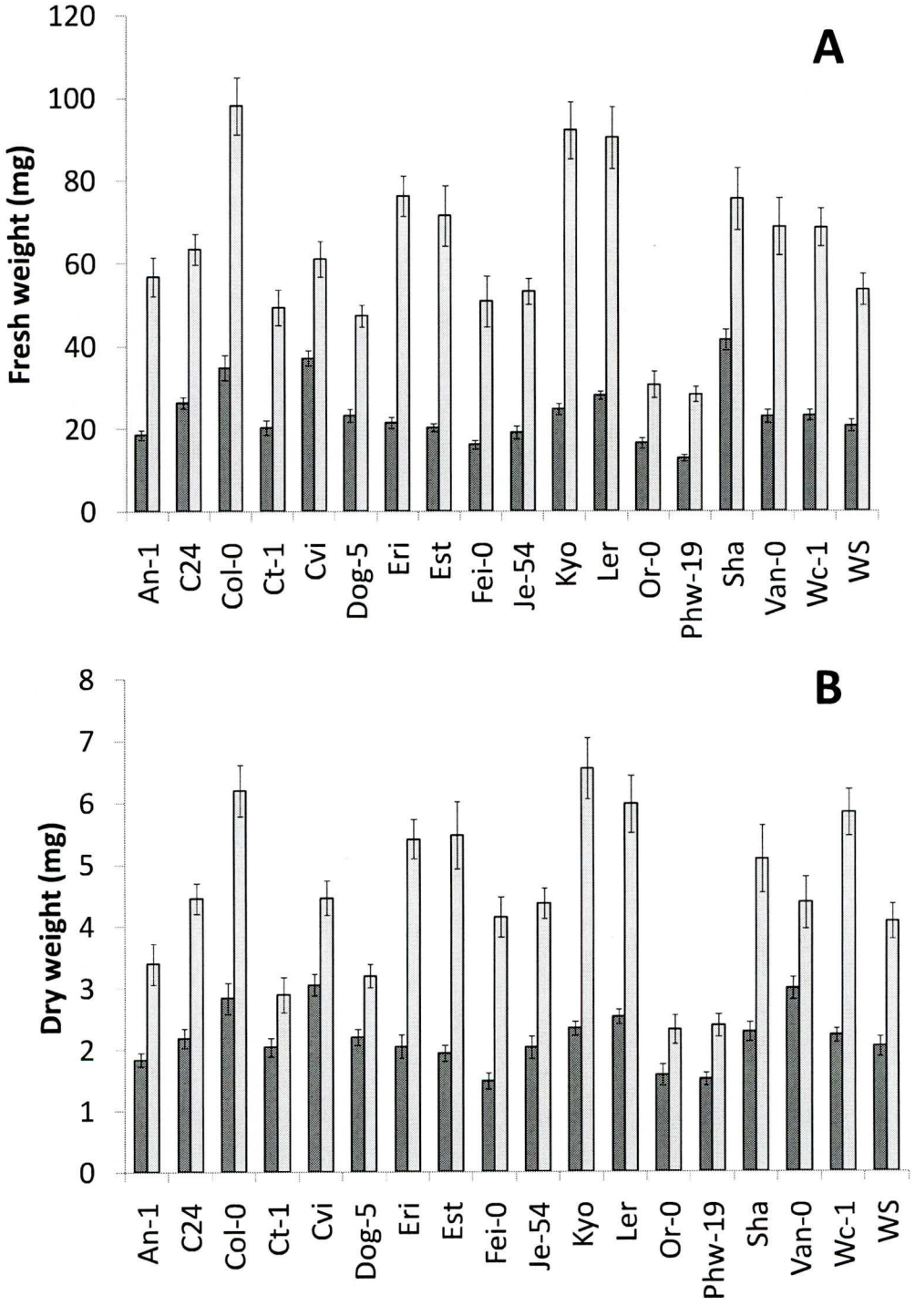

Figure 4.21. Natural variation in fresh (A) and dry (B) weight of *Arabidopsis* accessions measured at 17°C (dark bar) and 27°C (light bar).

Seedlings were grown at 22°C and 12:12 L:D for 12 days before transplanting to individual cell trays. After 2 additional days at 22°C, all trays were moved to 17°C or 27°C. All plants were harvested after 14 days and their fresh and dry weights were measured.

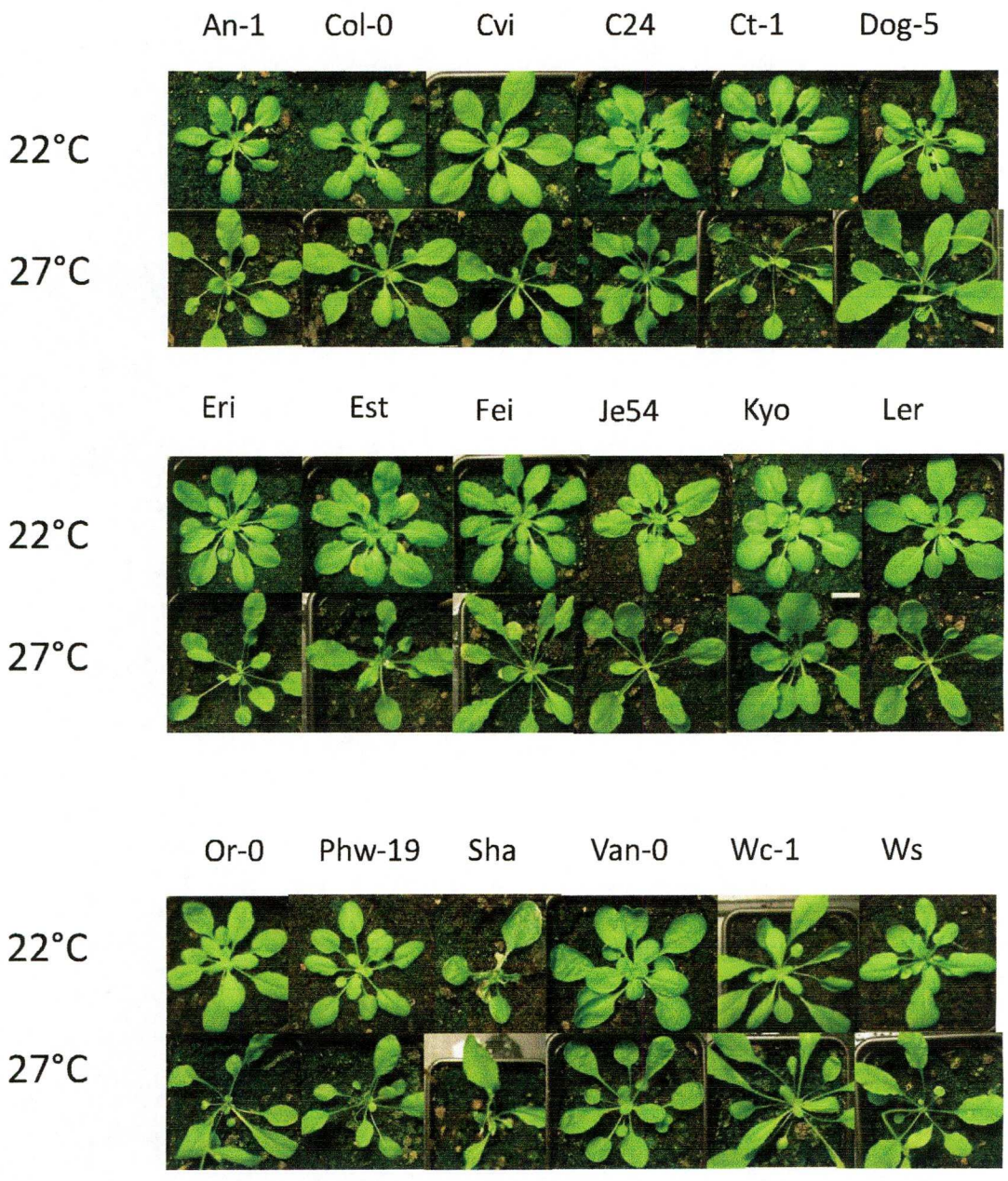

Figure 4.22. Examples of phenotypic variation between *Arabidopsis* accessions. Plants were grown for 29 days under 12:12 L:D conditions at either 22°C or 27°C.

Accessions were then ranked based on their weight gain value. According to the first growth performance experiment, subjection to 27°C for only 2 weeks resulted in plant weight gain ranging from approximately 0.75 mg to more than 4 mg dry weight (~15 mg to ~70 mg fresh weight). The growth of a few accessions was not affected appreciably and resulted in only a mild weight gain of less than 1 mg (Figure 4.23 B), which could indicate that such accessions are more resistant to the high temperature treatment. These accessions include Or-0, Ct-1, Phw-19 and Dog-5. On the contrary, Kyo, together with a few other accessions, were more sensitive to the 27°C and the increase in their weight reached more than 3 mg.

Interestingly, the degree of difference between fresh and dry weight gain and temperature varied in several accessions. For example, the mean weight for Van-0 increased considerably with the temperature, however ranking it by its dry weight increment placed it closer to the lower end of the weight gain range (Figure 4.23 A and B). On the other hand, for Sha the effect was opposite. This observation suggests that even though fresh and dry weights usually mimic each other, the relationship between the two in not always linear and other factors, such as differences in water retention capacity between plants/accessions, should be considered. However, overall, both fresh and dry weight gain was positively affected by the temperature increase, which was consistent across all accessions.

It has been shown that short and long period mutants grow better when under L:D conditions matching their endogenous clock (Dodd *et al.*, 2005), however, the effect of temperature compensation and plant performance has not been yet addressed. To determine if any connection between temperature compensation of the circadian clock and temperature dependent growth performance existed, a correlation

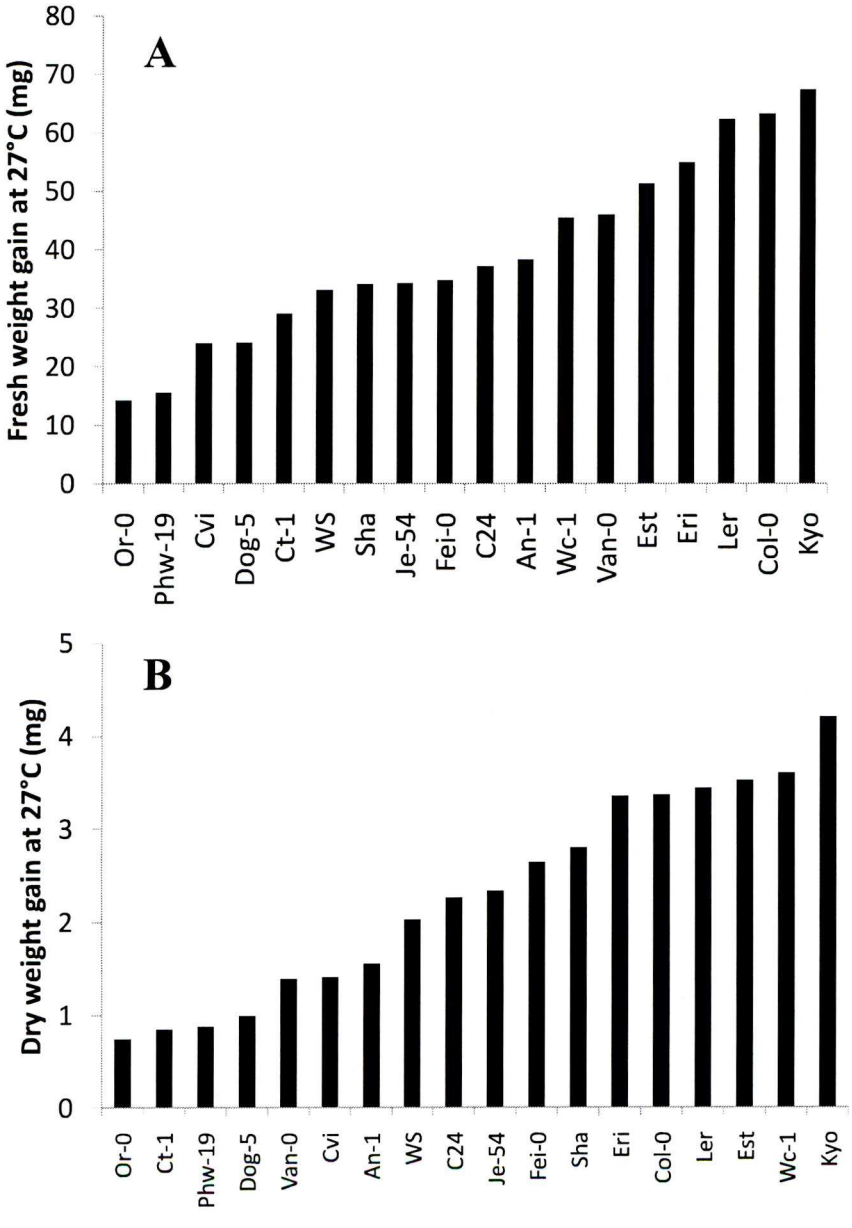

Figure 4.23. Natural variation in fresh (A) and dry (B) weight gain at 27°C for 18 *Arabidopsis* accessions.

Seedlings were grown at 22°C and 12:12 L:D for 12 days before transplanting to individual cell trays. After 2 additional days at 22°C, all trays were moved to 17°C or 27°C. All plants were harvested after 14 days and their fresh and dry weights were measured. Weight gain was calculated by subtracting the 17°C weight mean from the 27°C mean. Accessions are arranged from the smallest to the largest value.

was calculated between plant fresh and dry weight gain, and change in circadian clock outputs, i.e. leaf movement, *CCA1* and *LHY* expression. Both period change and change in RAE were examined. High temperature induced change in circadian outputs was calculated by subtracting the mean free running period (or RAE) estimated at 27°C from the 17°C mean estimate. This was done separately for all 18 *Arabidopsis* accessions. It was expected that accessions with the most temperature compensated circadian clock would be least affected by the temperature increase.

No correlation was found between the leaf movement period change and the weight change induced by the 27°C treatment (Figure 4.24 A and D). Such a result was somewhat unexpected as circadian leaf movement was the most temperature compensated process used in this study. Several accessions showed a well temperature compensated leaf movement phenotype (e.g. C24, Ct-1) (Figure 4.4 A), however, in terms of their weight gain, the response to the 27°C was very much accession specific (Figure 4.23). Furthermore, no correlation between the weight change and rhythm robustness of leaf movement was found, suggesting that temperature compensated leaf movement and plant growth could be independent from one another and failure of one would not necessarily be reflected by the other. On the other hand, it is more difficult to obtain accurate leaf movement data from plants grown at high temperature due to the elongated leaf petioles and fast growth of seedlings. Such seedlings fill up the space within a cell in just a couple of days, hence, proper movement of leaves becomes restricted affecting the accuracy of results. Therefore, interpretation of leaf movement data obtained from high temperatures should be viewed with caution.

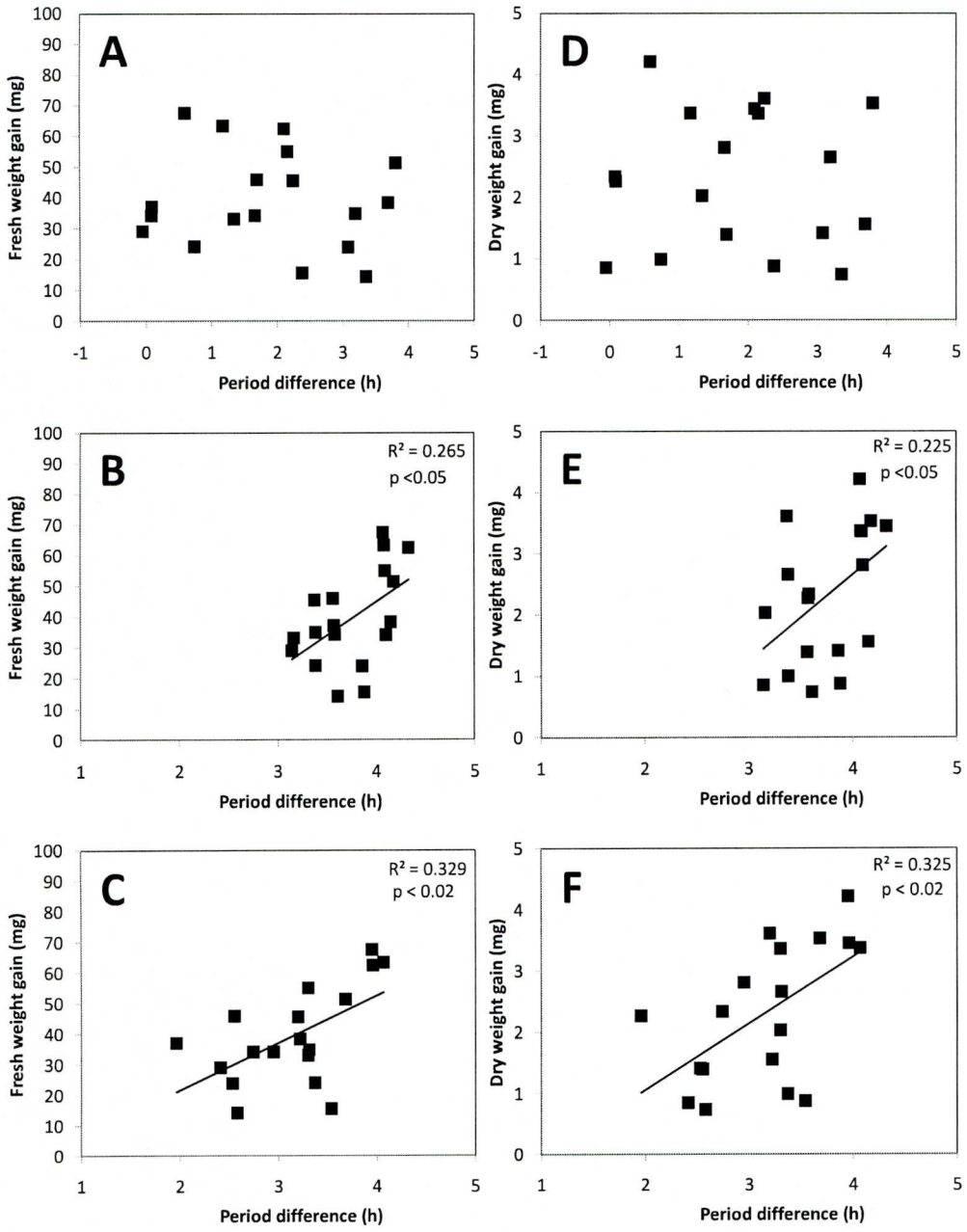

Figure 4.24. Correlation between growth performance and circadian period change in leaf movement (A, D), *CCA1* (B, E) and *LHY* (C, F) expression.

Free-running period of leaf movement, *CCA1* and *LHY* expression and fresh and dry weight measurements from 18 *Arabidopsis* accessions were obtained at 17°C and 27°C, and the means subtracted to get values for X and Y axes. Graphs on the left hand side represent correlation of fresh weight with circadian period change, while graphs on the right represent dry weight correlation. Weight values obtained from the first growth performance experiment were used.

Next, growth performance and circadian clock gene expression was investigated. Expression of *CCA1* and *LHY* should reflect an immediate change in circadian oscillator function, as they are members of the main feedback loop of the *Arabidopsis* clock. Therefore, correlation between *CCA1* and *LHY* expression and plant weight gain could provide an indication of whether *Arabidopsis* growth performance is somehow connected to temperature compensation of the clock. Significant positive correlation was revealed between the temperature-induced *CCA1* period change and the growth performance, with correlation coefficients of 0.515 for fresh weight and 0.470 for dry weight (Figure 4.24 B and E). The interaction between the weight gain and *LHY* period change with the temperature increase was even stronger (correlation coefficient of 0.573 and 0.570 for fresh and dry weight, respectively) (Figure 4.24 C and F). These results implied that accessions with a more temperature compensated clock (i.e. smaller period change between 17°C and 27°C) were least temperature responsive in terms of their growth. On the other hand, accessions with large period alterations also experienced large alterations in weight. No interaction between the growth performance change and rhythm robustness was found, which could be explained by the relatively minor change in the *CCA1* and *LHY* RAE, associated with the temperature increase (Table 4.2 and 4.3).

Unfortunately, no correlation between temperature compensation of the circadian clock and *Arabidopsis* weight change was found when the growth performance experiment was repeated (Figure 4.25). The light regime setting of the 27°C growth cabinet failed several times during the course of the second experiment resulting in a number of 24 h light or 24 h dark incidents.

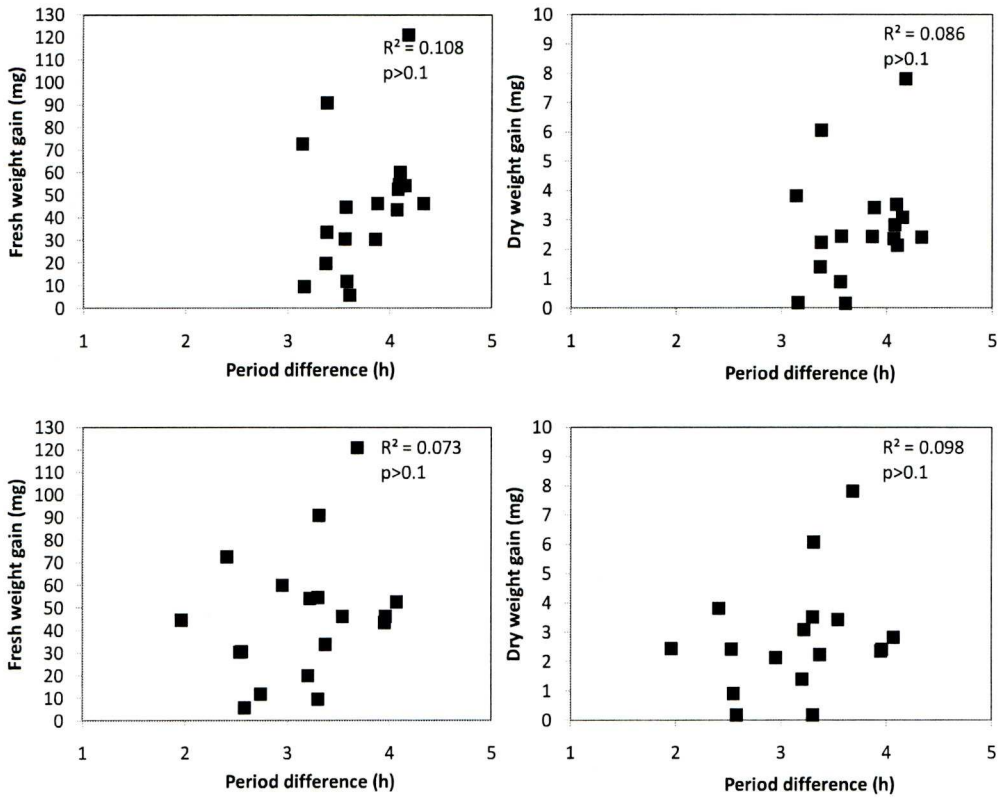

Figure 4.25. Correlation between growth performance and circadian period change in *CCA1* (A and C) and *LHY* (B and D) expression.

Free-running period of *CCA1* and *LHY* expression and fresh and dry weight measurements from 18 *Arabidopsis* accessions were obtained at 17°C and 27°C, and the means subtracted to get values for X and Y axes. Graphs on the left hand side represent correlation of fresh weight with circadian period change, while graphs on the right represent dry weight correlation. Weight values obtained from the second growth performance experiment were used.

Nevertheless, a connection between the naturally occurring temperature compensation of the circadian clock and plant growth performance, suggested by the first performance experiment, is highly possible. It makes sense for the accessions that have smaller changes in their circadian rhythmicity across a range of temperatures to be more temperature stable in other processes. Plant size and weight change could be one of these processes. Yet, the above proposed relationship should be further investigated and re-confirmed.

4.3. Discussion:

Natural variation in temperature compensation of the circadian clock was assessed in 18 *Arabidopsis* accessions by leaf movement assay and by monitoring expression of two circadian genes, *CCA1* and *LHY*. The current study reveals that while variation in leaf movement temperature compensation exists, all accessions experience period shortening in *CCA1* and *LHY* expression at high temperature. Analysis of 27°C and 33°C data demonstrates that as temperature increases circadian clock outputs are more likely to oscillate with different periodicities suggesting a temperature-induced uncoupling of the circadian clock loops. In addition, while it has been recently shown that a proper functioning circadian clock is beneficial for plants (Dodd et al., 2005), this study provides evidence that a well temperature compensated clock is of high importance in plant growth performance.

4.3.1. Natural variation in leaf movement

Analysis of leaf movement for 18 geographical accessions at 17°C and 27°C revealed considerable natural variation of free-running period in *Arabidopsis*. The majority of *Arabidopsis* accessions exhibited period shortening with the temperature increase, which is consistent with previous reports on temperature compensation (Edwards et al. 2005). Such period change was not surprising, as circadian rhythms are not entirely temperature independent. It is common to observe up to a few hours of period shortening when the temperature is increased 10°C or more (Gardner and Feldman, 1981; Onai et al., 2004; Edwards et al., 2005), however, this change is still minor considering the rate of physiological reactions under these conditions is expected to at least double (reviewed in Ruoff and Rensing, 2004). In any case, the degree of period shortening between the 17°C and 27°C temperature regimes was

accession dependent. In addition, the circadian rhythms of a few accessions were highly temperature compensated with no period change when measured at both temperatures. On the other hand, temperature also affects the robustness of the clock (Gould et al., 2006), and considerable variation was observed in this parameter. The robustness of the clock is a measurement of how well the data fits the cosine wave, where the lower the value, the better the fit and the more robust the rhythm is (Gould et al., 2006). Current results make it clear that temperature not only affects the period of the circadian rhythm, but also how well the clock oscillates. For example, in some accession, where the period was temperature insensitive, the clock robustness was decreased when assayed at the 27°C. Whilst for other accessions, despite displaying a period shortening at 27°C, the robustness of the rhythms was unaffected. Then again, several accessions exhibited both a period and a robustness change. Even though, no correlation between the temperature dependant period shortening and decrease in robustness was established, it is clear that both of these parameters should be considered when analysing circadian rhythms.

4.3.2. Natural variation in *CCA1* and *LHY* expression

In *Arabidopsis thaliana*, CCA1 and LHY are members of the main transcription/translation negative feedback loop of the circadian clock, and have been shown to be linked to temperature compensation (Alabadi et al., 2001; Gould et al., 2006). Therefore, both genes were chosen to assess the temperature-dependant variation in the *Arabidopsis* clock and temperature permissive range for circadian rhythmicity. Surprisingly, all accessions showed a strong period shortening with the temperature increase. The free-running rhythms of *CCA1* and *LHY* expression were altered by approximately 3-4 hours across all plants. In general, expression of both

genes were strongly interlinked and highly correlated in response to the increased temperatures (Figure 4.19), however some variation in period alteration was observed between different accessions. Several accessions exhibited period differentiation between *CCA1* and *LHY*, and this difference became more frequent at 27°C. The period difference between the two genes implies regulation from different oscillators. Indeed, several examples exist supporting the idea of the presence of several circadian clocks within a plant, generating different periodicities. For example, it has been demonstrated that *CAB* and *PHYB* (phytochrome B) oscillate with different periods even though both seem to be under similar circadian clock control mechanisms (Hall et al., 2002). Cytosolic free calcium (Ca^{2+}) and *Lhcb::LUC* have different free-running periods under constant red light conditions (Sai and Johnson, 1999). Furthermore, *CAT3* (*CATALASE 3)* and *CAB2* also display different periods specifically after entrainment to different temperature cycles, indicating that at least 2 oscillators with different temperature sensitivities are present within the plant (Michael et al., 2003b). It is possible that under normal conditions, multiple oscillators function in unison, however under certain circumstances, for example, large temperature change, these oscillators become uncoupled resulting in different periodicity in clock outputs. Multiple oscillators might also be created by uncoupling of negative feedback loops making up the central clock. Being partially redundant, it is not unlikely that CCA1 and LHY, members of the same loop under normal conditions, become functionally separated in an environment stressful for the plant, to regulate different circadian pathways. Due to variation in the geographical locations between *Arabidopsis* accessions, it is difficult to predict the degree of stress for each accession. Whilst 17°C is considered to be "normal" for common laboratory strains, it might be stressful for some "wild" *Arabidopsis* lines, and therefore may

result in an early uncoupling of circadian oscillators/loops explaining differentiation between *CCA1* and *LHY* in some accessions even when at 17°C.

Apart from temperature induced period shortening, the robustness of the *CCA1* and *LHY* rhythmicity was also affected, with *LHY* being affected more severely than *CCA1*, suggesting that transcription of *LHY* is more sensitive to the temperature increase. Gould et al. (2006) demonstrated that functional LHY is more important for *Arabidopsis* at higher temperature, while CCA1 is more important for lower temperature. Despite this temperature dependant differentiation, expression of *LHY* is highly down-regulated by the 27°C treatment in comparison to 17°C. Expression of *CCA1*, on the other hand, remains unaffected. Overall, it is intriguing how the importance of LHY increases with its increase in temperature sensitivity, suggesting that reduced *LHY* levels are essential in keeping the clock buffered against the heat stress. A straight decrease in LHY would undoubtedly cause a short circadian period phenotype (Alabadi et al., 2002), therefore, other proteins are highly likely to become involved in the process of keeping the whole circadian system balanced. One of these proteins has been proposed to be GI, which is a member of the evening loop of the central clock in *Arabidopsis* (Locke et al., 2005a). *GI* expression is up-regulated with the temperature increase and amounts of *LHY* are lowered in the *gi* null mutant, suggesting a temperature dependant connection between the two elements (Gould et al., 2006). It would be of interest to investigate variation in *GI* expression in different *Arabidopsis* accessions and examine if any correlation in the temperature dependant period change and robustness exists between *LHY* and *GI*.

In *N. crassa*, natural variation in the circadian period and temperature compensation has been linked to the length of the activation domain of the *WC-1*

gene, more specifically, to the number of simple sequence repeats (SSRs) present in the NpolyQ (amino-terminal polyglutamine domain) region (Michael et al., 2007). Even though variation in the correlation between the NpolyQ and circadian period in combination with the geographical origin exists, the general trend favours higher number of the NpolyQ SSRs in accessions closer to equator and with longer circadian rhythms. It is possible that *LHY* or/and *CCA1* are a source of phenotypic circadian variation in the temperature compensation in *Arabidopsis* accessions. It would be of high interest to identify if any elements are present within *CCA1* or *LHY* that could explain their molecular variation between different geographical accessions. Observed accessional differences in periodicity of *LHY* expression suggest that elements responsible for such variations could be linked to the *LHY* promoter. A recent discovery of the *FT* (FLOWERING LOCUS T) promoter mediating the variation in light-induced flowering between *Arabidopsis* accessions supports the fact that promoter elements influence natural variation in physiological responses and it is possible that the *LHY* promoter could be mediating the differences in circadian temperature compensation (Schwartz et al., 2009).

A number of studies attempted to correlate phenotypic and genetic parameters of *Arabidopsis* accessions with their geographical origins, however, so far, no strong correlation has been established (Pigliucci, 1998; Miyashita et al., 1999; Koornneef et al., 2004; Schmuths et al., 2004). Michaels et al. (2003) found that circadian period positively correlated with the day length calculated from the latitude of the accession's geographical origin, however, no link could be established between temperature compensation and the place where accession originates from, with the exception of the altitude (Edwards et al., 2005). It was speculated that this correlation was probably mediated by other factors specific to the micro-

environment, which in combination formed a very precise genotype. Nevertheless, no correlation was present between temperature compensation and expression of *CCA1* or *LHY*, with only one exception where the period of *LHY* correlated with the longitude. However, this association could be accidental and entirely based on a very low subset of accessions representing the far west and far-east, whereas the majority of accessions used in this study cluster around Eurasia. In addition, the same type of correlation was observed between leaf movement period, measured at 17°C, and longitude. As before, the link is very likely to be a result of under representation of accessions from certain geographical locations. However, if the correlation is indeed real, then it would be of interest to investigate the evolutionary advantages of the link between the periodicity and longitude.

Even though correlation between temperature compensation and some geographical coordinates was observed in the current study, this link was relatively weak. One of the reasons for this could be a relatively recent, largely human-facilitated, spread of *Arabidopsis*, with American and some Asian populations originating from Europe (Koornneef et al., 2004; Beck et al., 2008). On the other hand, the temperature dependant variation, found between accessions, suggest that circadian period and its buffering capability are adaptive traits possibly reflecting a very particular habitat with its very specific climate, including particular micro-temperature and light regimes (Stratton and Bennington, 1996; Reymond et al., 2007).

4.3.3. Temperature compensation and growth performance

A functional circadian clock is beneficial for *Arabidopsis* plant performance (Dodd et al., 2005), therefore, it is understandable that accessions with the smallest

temperature effect on their circadian phenotype i.e. better temperature compensated clock, would perform better when grown under elevated temperatures. In general, accessions with smaller *CCA1* and *LHY* period change between the 17°C and 27°C temperature experienced less fluctuation in their growth performance. However, this was not observed for the leaf movement rhythms, even though they were thought to be more temperature compensated. This suggests that the relationship between the temperature effect, circadian clock and performance is more complex than it was anticipated. Temperature influences plants in multiple ways, for example by affecting cell membrane fluidity, gene expression, protein structure, enzyme activity etc. (Law and Crafts-Brandner, 1999; Mikami and Murata, 2003; Matsuura et al., 2010), which consequently have an impact on physiological process, such as photosynthesis, growth and reproduction (Law and Crafts-Brandner, 1999; Tonsor et al., 2008; Zinn et al., 2010). The genetic variation between different *Arabidopsis* accessions could not only explain the differences in temperature compensation of *CCA1* and *LHY*, but also mediate the accession specific effect of the temperature on the link between the circadian clock and plant growth performance.

4.3.4. Natural variation in response to the 33°C treatment

Under the 33°C temperature condition, expression of *LHY* failed to oscillate and was down-regulated in all accessions regardless to their sensitivity or insensitivity to the 27°C treatment. In contrast, even though a few accessions abolished rhythmicity of *CCA1* expression, in several accessions it continued to oscillate despite a high reduction in the rhythm amplitude. A role switch between the CCA1 and LHY importance in temperature compensation, during the temperature increase from 27°C to 33°C, has recently been proposed (A. Hall, pers. commun.). It

becomes apparent that while transcription of *LHY* becomes irrelevant at such an elevated temperature, *CCA1* takes up the role of driving circadian rhythms, most likely in combination with other clock components, e.g. PRRs and GI (Salome and McClung, 2005; Gould et al., 2006), supporting the idea of uncoupling of the clock loops. It would be interesting to investigate if other clock loops are still rhythmic and are capable of driving the overt rhythms. Unfortunately, analysis of circadian rhythms at 33°C using leaf movement is not reliable due to the increased rates of seedling growth, where new leaves emerge almost every 24 h thus obstructing a clear view of the first leaf pair. Furthermore, delayed fluorescence has not yet been adapted for usage at high temperatures, and had not been generating reliable data. Therefore, a screen for expression of genes related to the circadian clock at 33°C would provide a more useful tool to determine the functioning of the oscillator at elevated temperatures.

Interestingly, while at 27°C all accessions experienced a period shortening in *CCA1* expression, accessions where *CCA1* still oscillated under the 33°C treatment, exhibited period lengthening. However, a large degree of variation in this temperature induced period change was present with accessions displaying rhythms of considerably shorter than 24 h, around 24 h, and longer than 24 h period (Table 4.5). This suggests that accession dependant variation exists in the mechanism driving rhythmicity of *CCA1*, and it is highly possible that an oscillating clock has been under selective pressure from some environmental factors specific to their environment. Unfortunately, detailed information about collection sites for *Arabidopsis* accessions used in this study is lacking, therefore, at this moment it is not possible to examine correlations between *Arabidopsis* clock parameters and the

environment each accession arose from, and to propose the ultimate factor influencing the functioning of the circadian oscillator.

4.3.4. Effect of the 33°C temperature treatment on *Arabidopsis* circadian phenotype

Temperatures of 30°C and above are stressful for *Arabidopsis*, especially if the heat exposure is prolonged (Tonsor et al., 2008; Wienkoop et al., 2008; Zinn et al., 2010). To examine whether 33°C treatment was highly damaging for *Arabidopsis* plants with irreversible damage to plant physiology and consequently a breakdown of the circadian system, expression of *CCA1* and *LHY* was monitored in seedlings exposed for 3 days to 33°C followed by a temperature switch back to 17°C. As expected, all accessions abolished their rhythmicity in *LHY* expression, and only accessions, previously mentioned as *CCA1* rhythmic at 33°C, retained their oscillations in the *CCA1* transcription (Figure 4.17). After the temperature was reduced to 17°C, the rhythmicity and amplitude for both *CCA1* and *LHY* was regained (Figure 4.18), suggesting that high temperature only inhibits these circadian rhythms, but does not permanently disturb them. Despite the differences in *CCA1* periodicity under 33°C conditions, a temperature shift caused the circadian clock to reset, so that all accessions reinitiated their rhythms at the identical phase. High and low temperatures have been known to "hold" circadian rhythms, and a switch to normal conditions re-starts the rhythms with or without a phase shift (Njus et al., 1977; Grams et al., 1997; Rensing and Ruoff, 2002). Temperature fluctuations affect the CO_2 exchange rhythm in *Kalanchoe tubiflora* (Grams et al., 1997). Laboratory experiments in combination with modelling show that continuous exposure of *Kalanchoe* plants to 30°C "freeze" the circadian rhythmicity of CO_2 exchange,

whereas a temperature switch from 30°C to 20°C reinitiates the rhythm with the phase corresponding to the temperature change (Grams et al., 1997). Interestingly, while low temperature also "holds" the circadian rhythm, a switch back to the normal temperature restarts the rhythm with a 180° phase shift (Grams et al., 1997). Circadian rhythms of *CAB* transcription in tomato seedlings are abolished at 30°C under continuous light, however, it continues to oscillate and L:D conditions (Riesselmann and Piechulla, 1990). It would be interesting to investigate if this is also the case for *Arabidopsis*.

Overall, examination of 33°C *CCA1* expression in different *Arabidopsis* lines implies that the range permissive for *CCA1* rhythmicity is highly accession dependant. While in some accessions, *CCA1* expression oscillates at 33°C, in other the circadian clock stops and the oscillations are completely abolished. However, when the plants are returned to permissive temperatures, the clock starts up again generating rhythms (Njus et al., 1977). On the other hand, it is possible that a failure to detect circadian oscillations is due to the uncoupling between circadian loops of the clock (Njus et al., 1977; Hall pers. commun.), hence while in some accessions *CCA1* is still functionally driving low amplitude rhythms, in the rest of the accessions rhythms are already being driven by less temperature sensitive elements. Expression analysis of other circadian genes would provide information for more conclusive data interpretation. Nevertheless, 33°C is a stressful temperature for *Arabidopsis* and judging by the poor appearance of seedlings exposed to the continuous 33°C temperature for more than 5 days (data not shown), it is highly probable that plants will be damaged permanently and the loss of circadian rhythms will be irreversible.

The variation in the upper limit of the temperature range permissive for circadian rhythms, as well as variation in circadian clock robustness between accessions, might be mediated by heat shock proteins (HSP), which are linked to *Arabidopsis* performance and reproductive fitness under heat stress (Queitsch et al., 2000; Queitsch et al., 2002; Tonsor et al., 2008). Variation exists in the levels of the HSP 101 (Heat shock protein 101) protein which has been linked to variation in thermotolerance of *Arabidopsis* (Tonsor et al., 2008). Accession thermotolerance was assessed by measuring plant performance and fitness after a number of 35°C exposure periods (Tonsor et al., 2008). Elevated temperatures increase the expression of HSP 101 in the Col-0 accession, however its expression is constantly low and temperature insensitive in the Ler background. Interestingly, the performance and fitness of the Col-0 plants lacking a functional HSP 101 gene is negatively affected by the high temperature treatment, whereas performance of the Ler plants does not change with or without the HSP allele (Tonsor et al., 2008). Analysis of the HSP 101 link with other phenotypic traits such as root growth, hypocotyl elongation and drought tolerance, suggests the pleitropic nature of the heat shock proteins. Taking that into consideration, it is possible that these proteins also interconnect with the circadian clock. Maybe HSP- dependant thermotolerance in Col-0 could explain the rhythmic phenotype in *CCA1* expression under the 33°C conditions, whereas low expression of the HSP 101 in Ler could be linked to the 33°C induced suppression of *CCA1* and *LHY*. Further examination of the link between the HSP mediated thermotolerance and the temperature compensation of the circadian clock across multiple accessions would be a valuable contribution to the further understanding of the complexity of plant responses to changes in the environment.

Chapter 5 – General discussion:

Plants lacking either CCA1 or LHY have a short period phenotype suggesting that these proteins are only partially redundant and cannot completely substitute one another (Green and Tobin, 1999; Alabadi et al., 2002). Therefore, presence of both elements is necessary to have a balanced oscillator which controls circadian outputs properly. The current work offers further support to this statement. Analysis of transgenic plants expressing *CCA1* and *LHY* from the opposite promoters shows that the *LHY*::*CCA1* construct is capable of restoring CCA1 activity in *cca1*- null plants. On the contrary, *lhy* null plants carrying *CCA1*::*LHY* developed a very short period phenotype, mimicking a *cca1*:*lhy* double mutant. The nature of this phenotype is unclear, however, it has recently been suggested that other negative feedback loops could partially substitute the loss of members of the main clock loop (Lu et al., 2009). Nevertheless, the reason for the suppression of *CCA1* and *LHY* transcription remains unknown. It is possible that in the absence of *CCA1* a constitutive repressor is present on the *CCA1* promoter, which is usually outcompeted by autoregulation by CCA1. Creation of *CCA1*::*LHY*-in-*lhy* null plants where transcription of *LHY* is driven by the *CCA1* promoter offers a basis for a possible theory that the enhanced inhibitor of the *CCA1* promoter could be LHY. *lhy* null and *cca1* null produce robust circadian rhythms. *LHY*::*CCA1*-in-*cca1* null plants were also able to maintain robust rhythms and keep them up close to the wild type levels. This suggests that while *LHY* promoter can substitute for the absence of the *CCA1* promoter, it does not work the other way around and when both functional genes are present the *LHY* promoter is required to ensure the circadian system is in balance.

Inhibition of *CCA1* transcription could be due to possible differences in the degree of affinity to the *CCA1* promoter between CCA1 and LHY homo/hetero

dimers. CCA1 and LHY can have activator and repressor functions (Wang and Tobin, 1998; Kawamura et al., 2008; Nakamichi et al., 2010), and it is possible that this function depends on whether the two proteins homo- or heterodimerize. For example, while LHY homodimers repress transcription of *CCA1*, CCA1/LHY heterodimers could promote it. Working in parallel, both products could ensure the correct functioning of the clock. On the other hand, both *cca1* and *lhy* single mutants have a short period, implying that both genes are needed for a normal rhythm, which suggests that heterodimerization is of primary significance. This hypothesis should be further investigated.

In addition, the *LHY*::*CCA1*-in-*cca1* null phenotype is temperature compensated, while *CCA1*:: *LHY* transplants become arrhythmic at 27°C. It is possible that whatever gene/protein loops were able to partially substitute CCA1 and LHY at normal temperature, their function was abolished when the temperature was increased. Another interesting observation is an increase in *CCA1* and *LHY* transcription in *CCA1*::*LHY* lines when subjected to 27°C, which is the opposite of that observed in the rest of the transplants analyzed in this study. As suggested earlier, if *LHY* is highly recruited to the *CCA1* promoter and increased temperature negatively affects *LHY* expression, this should subsequently result in a temporary release of the *CCA1* suppression and an increase in the *CCA1* mRNA. Plants, where production of CCA1 and LHY is not completely eliminated, have an arrhythmic phenotype (Lu et al., 2009), as also observed in this study. It would be of interest to investigate whether introduction of the *LHY::CCA1* construct into *CCA1::LHY* lines would be able to restore proper circadian phenotype in these plants.

Analysis of plants carrying extra copies of either *CCA1* or *LHY* showed that *Arabidopsis* plants are capable of retaining circadian rhythms even when amounts of

CCA1 or *LHY* mRNA are elevated several fold. In addition, large variation in *CCA1* and *LHY* mRNA amounts between the individual transgenic lines did not result in the variation in any other clock outputs, i.e. leaf movement or delayed fluorescence period. Overall, it is clear that the circadian clock mechanism represents a highly buffered system and as long as some essential regulation elements for each gene are present, the system stays balanced. It is possible that different mechanisms of regulating *CCA1* and *LHY* on transcript and protein levels ensure a tight control of the circadian oscillator under continuously fluctuating environmental conditions.

The difference between *CCA1* and *LHY* was further supported by analysis of the temperature dependent variation of circadian period and robustness across *Arabidopsis* accessions. The 27°C treatment had a considerable effect on *CCA1* and *LHY* expression, causing a shortening of the free running period in all plants. Despite that, the degree of period shortening was accession dependant and was more variable in *LHY*, which has previously been shown to play a more significant role than *CCA1* in buffering the circadian clock against high temperatures. Hence, it will be important to identify the elements influencing differentiation in *LHY* expression across accessions. Judging from the analysis of the promoter switch lines, it is possible that *LHY* is involved in the temperature sensing of *Arabidopsis* either directly or through components upstream or downstream of its regulation. Molecular polymorphism of these components in combination with the potential differences in the *LHY* promoter could contribute to the accession specific response observed in this study.

In addition, the temperature compensation of leaf movement also differed between accessions. Despite the universal reduction in the *CCA1* and *LHY* period length at the high temperature, the period for leaf movement in some accessions

remained unchanged. This data implies that uncoupling of the circadian loops occurs and other elements become responsible for driving separate oscillations resulting in outputs of different periodicities. No straightforward link between the circadian outputs and their response to temperature, valid across all accessions, could be established, suggesting that different systems/pathways, unique for each individual background, have probably been established as a buffering mechanism against the temperature changes. These unique mechanisms ensure the optimal performance for each accession genotype in their unique microenvironment. Overall, it has been confirmed here that the temperature effect is pleiotropic and could not so far be evaluated by measuring only 1 parameter.

Subjection of plants to the 33°C treatment caused a down-regulation of *CCA1* and *LHY*. However, while at 33°C *LHY* expression became arrhythmic in all plants, in selected accessions *CCA1* continued to oscillate. This result confirms that the temperature range permissive for rhythmicity is variable across accessions. Temperature largely influences plant performance and resistance to heat stress, which is especially crucial in commercially grown plants (Boyer, 1982; Swindell et al., 2007), hence the identification of the stress related components and their role in the temperature response is essential, especially in relation to the increase in the global temperatures (Cox et al., 2000). The significance of a functional circadian clock to plant performance and survival has previously been shown (Dodd et al., 2005), and, therefore, it is not unreasonable to assume that a functional clock is also important when plants are under stress. The current study provides an attempt to establish a link between the temperature compensation of the circadian clock components and temperature treated plant performance. It is clear that a more extensive investigation is needed to further advance our understanding of the role of

the clock and its physiological functioning in improving the tolerance of plants to temperature stress. Identification of the naturally present components contributing to stress tolerance could not only expand the geographical range over which certain valuable plants/crops can grow, but could also help bypass the increasing public concern for the growing practice of the transgenic plants.

The understanding of the CCA1 and LHY role in temperature compensation is of primary importance, as homologues of *Arabidopsis* CCA1 and LHY genes are found in other dicot (e.g. poplar) and monocot plants (e.g. rice and barley) (Izawa et al., 2003; Cotter et al., 2010). Interestingly, the number of *CCA1* and *LHY*- like genes is different depending on a plant. While poplar and *Lemna* species have 2 copies of the gene, *Oryza sativa* (rice) possesses just one (Miwa et al., 2006; Murakami et al., 2007). It has been shown that both poplar genes, *PnLHY1* and *PnLHY2* rhythmically oscillate and expression of the *PnLHY2* is considerably higher than of the *PnLHY1* (Takata et al., 2009). Expression of *Arabidopsis LHY* is also higher than of the *CCA1* (Lu et. al., 2009 and this study), suggesting some functional and regulatory similarities might exist between the two plant species. In addition, it is curious to speculate that the function of *PnLHY1* and *PnLHY2* while redundant under normal conditions have differential roles under stress. In contrast, the temperature compensation mechanism is most likely to be divergent in plants with only 1 copy of the *CCA1/LHY* gene. In *Neurospora,* buffering of the circadian clock against temperature changes is mediated by alternative splicing of *FRQ* (Liu et al., 1997). The *HvCCA1* (barley *CCA1*) transcript also has an alternative splice variant (Cotter et al., 2010), which could be highly significant in stress mediated responses.

Overall, the genetic approaches used in this study suggest that the temperature compensation mechanism of the circadian clock in *Arabidopsis* is very

complex. On the other hand, it has been recently demonstrated that 3 main circadian proteins isolated from cyanobacteria are able to maintain oscillations in a test tube and are intransigently temperature compensated (Nakajima et al., 2005), implying that post-translational protein regulation could be a major factor in the temperature compensation mechanism. Further studies incorporating protein interaction and their post-translational regulation would provide inimitable information towards understanding the circadian system and its properties.

Future perspectives:

- The *Arabidopsis* mathematical model, which is based on experimental data from the 22°C condition, combines the functions of the CCA1 and LHY under the assumption that they share same role. More data is starting to emerge, including from this study, that the 2 genes do not always have the same function. Furthermore, it is becoming apparent that the clock system can alter depending on environmental conditions. This new data should be incorporated into the existing bank of experimental data and the clock model should be updated accordingly.
- Switching promoters between *CCA1* and *LHY* resulted in plants with different phenotypes, suggesting that these circadian components are not entirely redundant. It is of great interest to investigate why the *LHY::CCA1* construct was capable of complimenting *cca1* null plants while *CCA1::LHY* failed to rescue *lhy* null. One way to approach the issue would be to examine the affinity of CCA1 and LHY to their own and each other promoters. The promoter affinity might also differ depending on whether the proteins form homo- or hetero-dimers.

- In the future it would be interesting to examine importance of CCA1/LHY homodimerization in promoter regulation by examining *lhy* null plants transformed with the *LHY::CCA1* construct and *cca1* null plants carrying *CCA1::LHY* construct. These plants were created, however, not analyzed in this study due to the time constraint.
- Plants carrying extra copies of *CCA1* and *LHY* responded differently to the temperature increase. The 27°C period increase observed in the *LHY::LHY*-in-*Ws* plants in comparison with the no period change in *CCA1::CCA1*-in-*Ws*, could imply that LHY plays a role in prolonging circadian rhythms when the temperature increases. It would be interesting to compare the gene expression of *CCA1* and *LHY* in both types of plants at 27°C as well as further investigate the separate and combined dosage effect of the two genes on the circadian system.
- There is natural variation in temperature compensation in *Arabidopsis* accessions with more variation in *LHY* expression than in *CCA1*. Several accessions with the most pronounced differences could be selected to identify the cause of the differential temperature response between ecotypes. Analysis of elements known to interact with LHY as well as to be involved in the temperature compensation response, e.g. GI, should be a priority task.
- An uncoupling of circadian outputs occurred when *Arabidopsis* plants were subjected to high temperature, however, the degree of uncoupling differed between accessions. It is important to understand the significance of the uncoupling process and investigate its impact on the overall performance of plants. The current study suggests that plants with the least change in *CCA1*

and *LHY* are more tolerant to temperature stress, however, more work is needed to ensure this hypothesis is fully correct.

- Analysis of plants under 33°C conditions confirms that some accessions are more tolerant to the temperature stress than others. A complete arrhythmia in *LHY* expression, in comparison to rhythmic oscillations in *CCA1* expression, suggests that different components could be required for proper functioning of the clock at 33°C. These components should be identified. In addition, the potential physiological significance of the functional circadian clock at such elevated temperatures should be investigated, as this knowledge could be used in extending the temperature range over which plants, including commercially important crops, can flourish.

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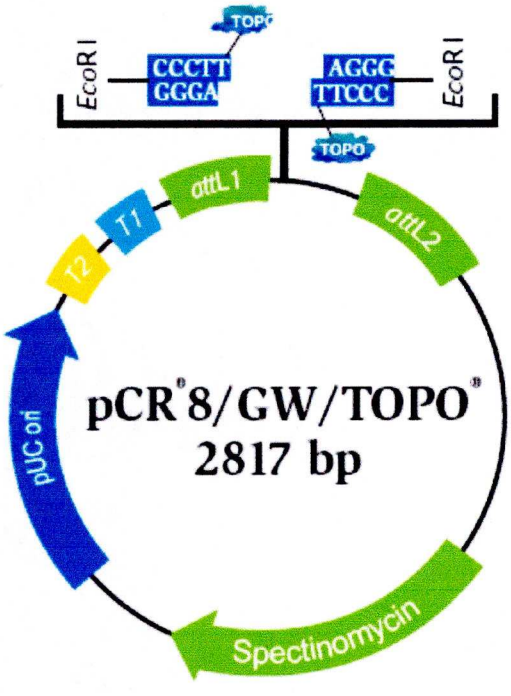
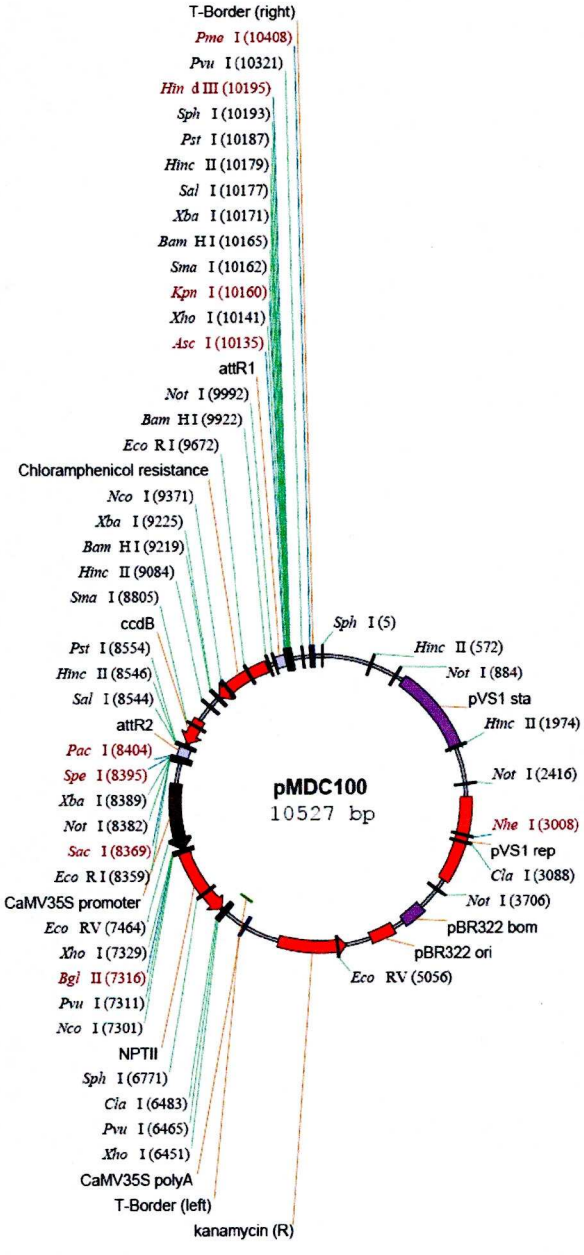
Supplementary data:

Appendix 1. Plasmid map of pCR®8/GW/TOPO® vector, from http://tools.invitrogen.com/content/sfs/vectors/pcr8gwtopo_map.pdf



Appendix 2. Gateway plant binary vector pMDC100, from
http://botserv1.uzh.ch/home/grossnik/curtisvector/pMDC100.pdf



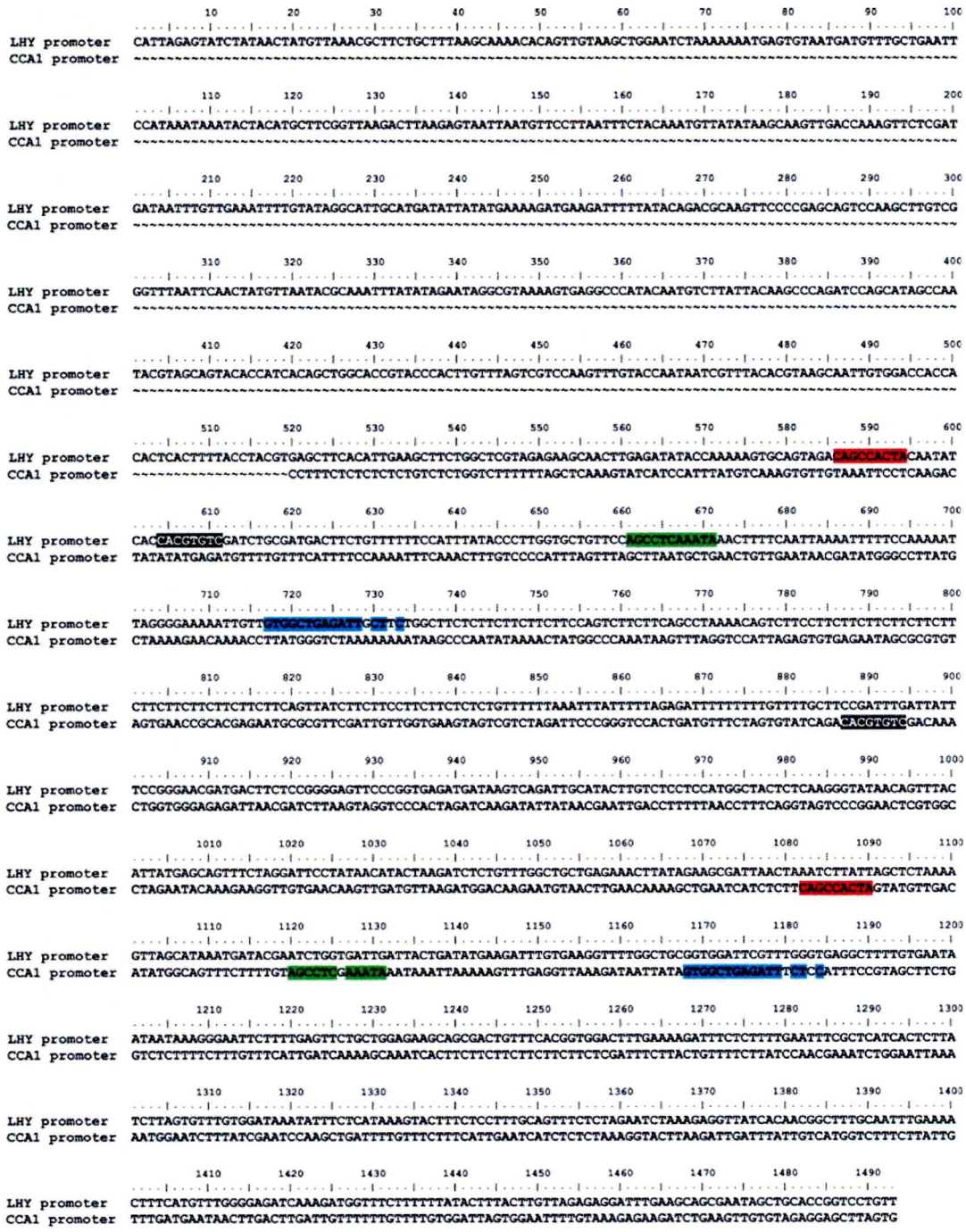


Figure S1. Comparison of *CCA1* and *LHY* promoter regions.

G – box is shown as a black box with white letters; coloured boxes are conserved sequences found in both promoters: red box – conserved region 1, green box – conserved region 2 and blue box – conserved region 3 (modified from Spensley et al., 2009).

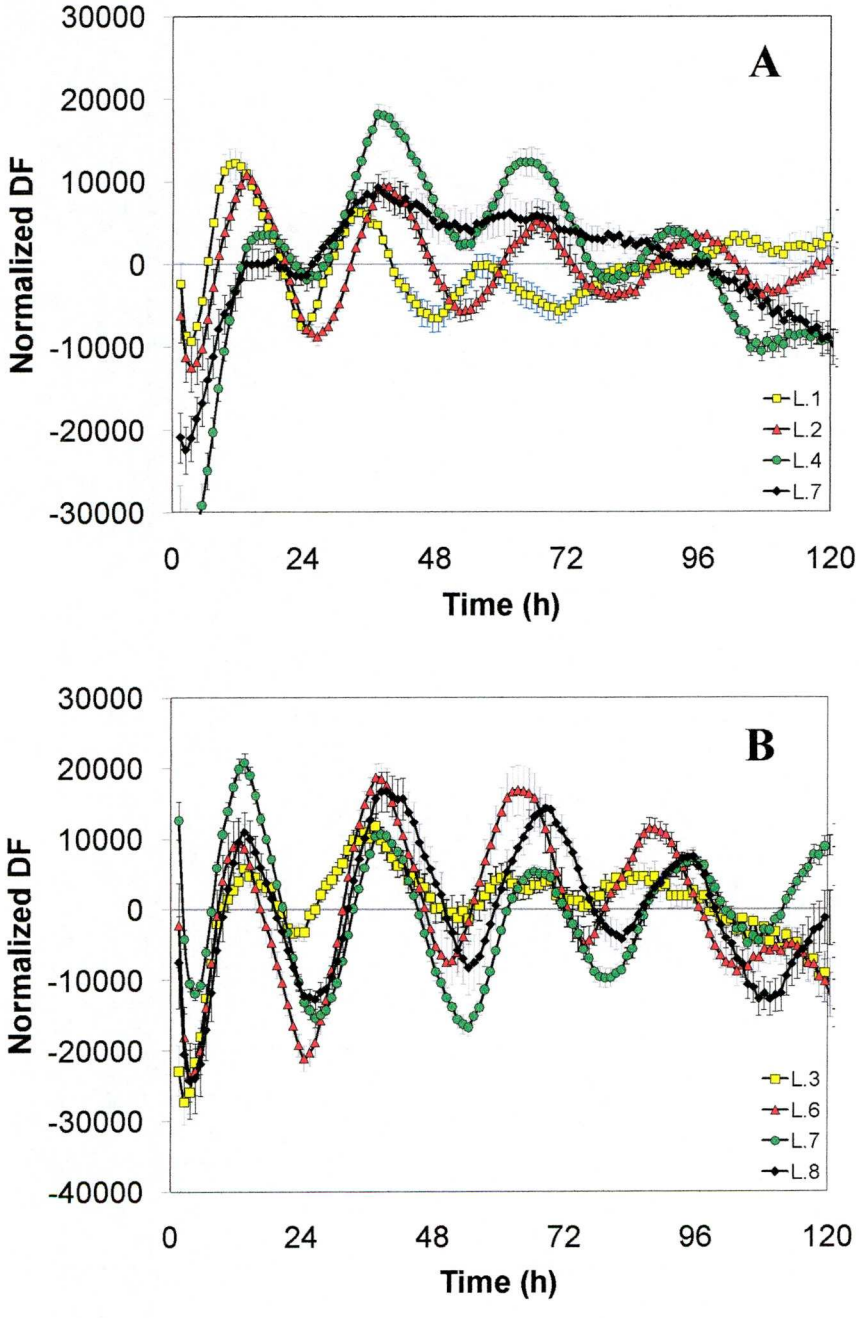


Figure S2. Delayed fluorescence rhythms for *CCA1::CCA1* (A) and *LHY::LHY* (B) transgenic lines.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 15 days before the transfer to continuous light, where delayed fluorescence was monitored, n=8. The plots show normalized averages (+/- SE) for DF in 4 representative transgenic lines.

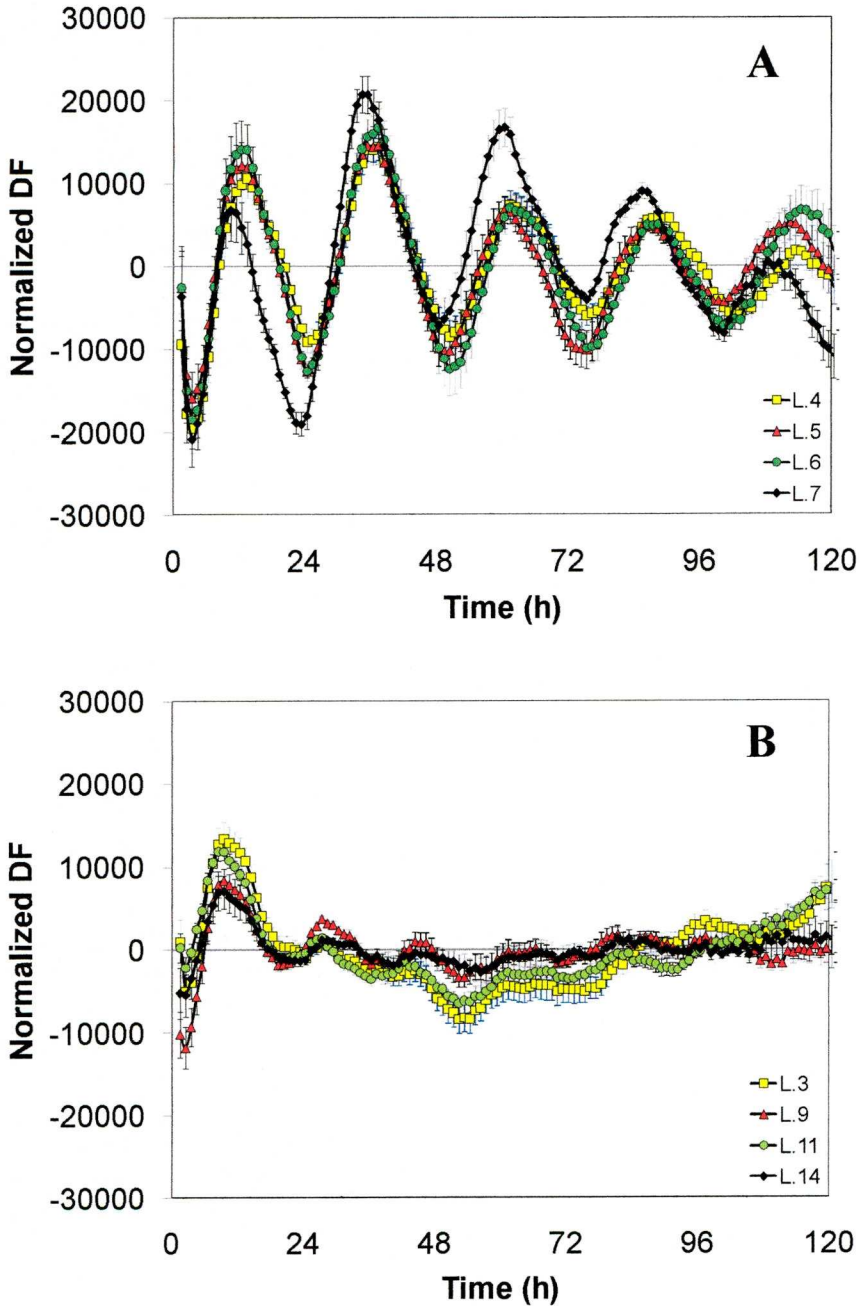


Figure S3. Delayed fluorescence rhythms for *LHY::CCA1* (A) and *CCA1::LHY* (B) transgenic lines.

Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 15 days before the transfer to continuous light, where delayed fluorescence was monitored, n=8. The plots show normalized averages (+/- SE) for DF in 4 representative transgenic lines.

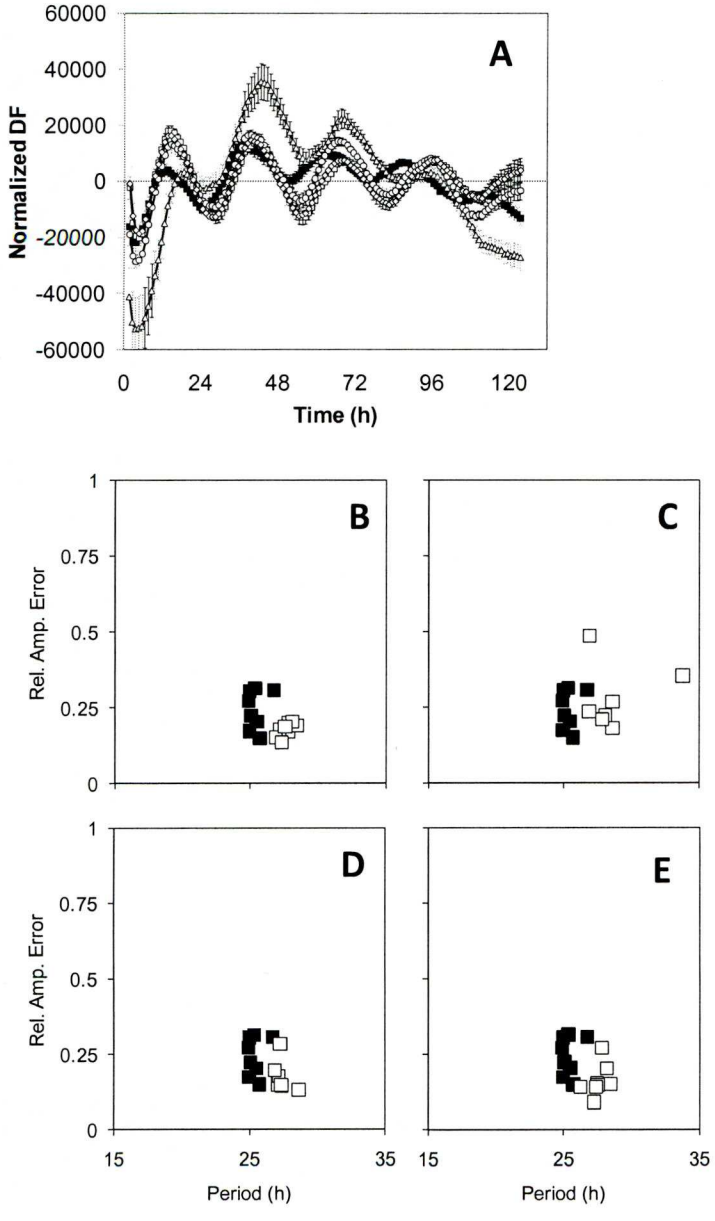


Figure S4. Delayed fluorescence rhythms for *CCA1::CCA1*-in-Ws transgenic lines. Introduction of additional *CCA1* to Ws causes a mild increase of the circadian period measured by delayed fluorescence.

A – represents normalized averages +/- SE for delayed fluorescence in Ws (black squares) and *CCA1::CCA1*-in-Ws transgenic lines 2 (empty squares), 4 (empty triangles), 10 (empty circles) and 12 (empty diamonds). B, C, D and E – Circadian period estimates for delayed fluorescence plotted against Rel. Amp. Error. Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 15 days before the transfer to continuous light, where delayed fluorescence was monitored, n=8. All experiments were independently repeated at least twice with similar results.

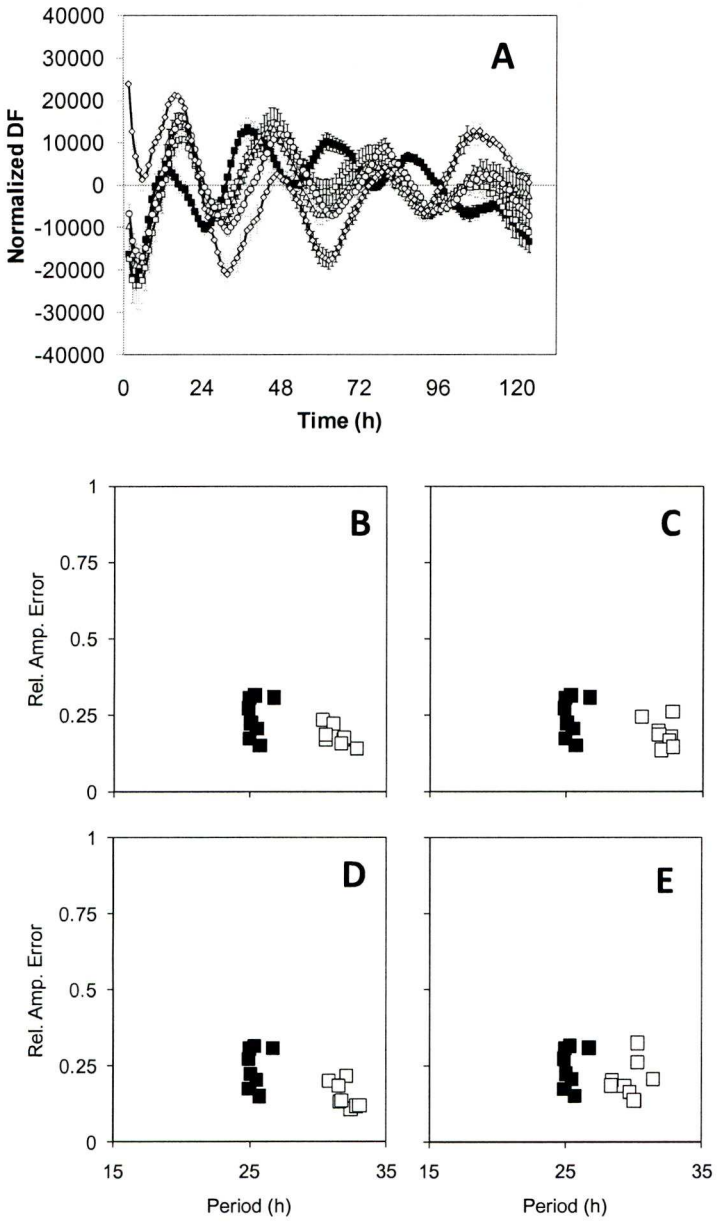


Figure S5. Delayed fluorescence rhythms for *LHY::LHY*-in-Ws transgenic lines. Introduction of additional *LHY* to Ws causes a considerable increase in the circadian period measured by delayed fluorescence.

A – represents normalized averages +/- SE for delayed fluorescence in Ws (black squares) and *LHY::LHY*-in-Ws transgenic lines 1 (empty squares), 2 (empty triangles), 4 (empty circles) and 8 (empty diamonds). B, C, D and E – Circadian period estimates for delayed fluorescence plotted against Rel. Amp. Error. Plants were grown on MS agar supplemented with 3% sucrose under 12:12 L:D for 15 days before the transfer to continuous light, where delayed fluorescence was monitored, n=8. All experiments were independently repeated at least twice with similar results.